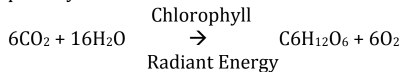
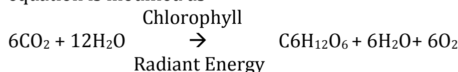
A. Photosynthesis: Light harvesting complexes; mechanisms of electron transport; photoprotective mechanisms; CO_2 fixation-C_3, C_4 and CAM pathways.

Photosynthesis is essentially the only mechanism of energy input in the living world. Photosynthesis (photos-light, synthesis-putting together) is an anabolic process of manufacture of organic compounds inside the chlorophyll containing cells from carbon dioxide and water with the help of sunlight as a source of energy. A simple equation of photosynthesis is as follows:



$$6CO_2 + 16H_2O \xrightarrow[\text{Radiant Energy}]{\text{Chlorophyll}} C6H_{12}O_6 + 6O_2$$

However, the function of water is to provide hydrogen for the synthesis of organic compounds.
All the liberated oxygen comes from it. Therefore, the equation is modified as



$$6CO_2 + 12H_2O \xrightarrow[\text{Radiant Energy}]{\text{Chlorophyll}} C6H_{12}O_6 + 6H_2O + 6O_2$$

The source of molecular oxygen was water and not carbon dioxide as was believed earlier was experimentally proved first by Robert Hill (1937) and later confirmed by M.D. Kamen, and S. Ruben (1945), employing tracer technique in which heavy isotopes of oxygen ^{18}O were used. But this experimental proof was based on the suggestions of C.B. Van Niel's work on bacterial photosynthesis (1930). He suggested that in green plants H_2O is the source of reduction and when split yields (H) and (OH), and O_2 released by plants is derived from water, not from CO_2. This splitting of water in light by green plants has come to be known as photolysis of water and the theory, Van Niel's theory of photolysis of water.

Chloroplasts: Structures and Photosynthetic Pigments

Chloroplasts is the seat of photosynthesis and is best exemplified in the higher plants. A chloroplast is covered by an envelope of two membranes which are separated by periplastidial space of 10-20 nm. Internally a chloroplast contains a matrix or stroma in which are embedded a number of flattened membranous sacs called thylakoids or lamella. The external surface of thylakoids contains the photosynthetic pigments and serves the ends of light re-action. The stroma, on the other hand, is concerned with the events of the dark reaction. In certain regions the thylakoids are stacked to form grana. The longer thylakoids that connect one granum to another extend through the stroma so these membranes are usually referred to as stroma thylakoids.

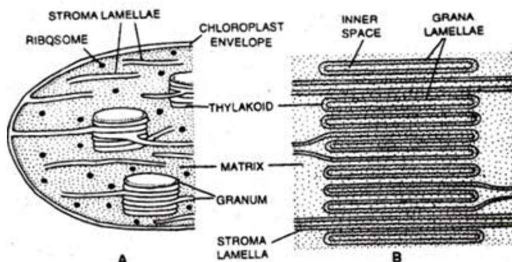
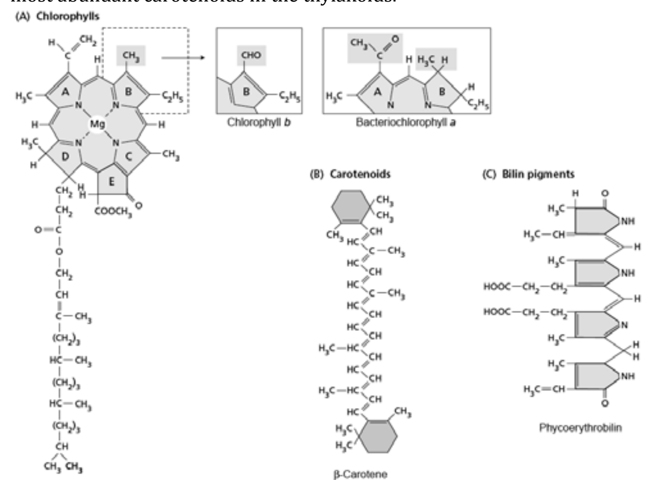


Fig. : Chloroplast thylakoids. A, part of the chloroplast showing stroma thylakoids and grana; B. arrangement of thylakoids in a granum.

Photosynthetic Pigments: The photosynthetjc pigments present in thylakoid membranes consist largely of two kinds of green chlorophylls, Chlorophyll a ($C_{55}H_{70}O_5N_4Mg$) and Chlorophyll b ($C_{55}H_{72}O_6N_4Mg$). Also present are yellow to orange pigments classified as carotenoids. There are two kinds of carotenoids, the pure hydrocarbon carotenes and the oxygen-containing xanthophylls. Certain carotenoids, especially violaxanthin, a xanthophyll, also exist in the chloroplast envelope, giving it a yellowish colour. In most plants, including green algae, β-carotene and lutein are the most abundant carotenoids in the thylakoids.



Events of Photosynthesis

Photosynthesis consists of two types of reactions: a light dependent one and a light independent one. The light-dependent reaction is a photochemical reaction or light reaction as it came to be called, culminating in the generation of NADPH2, ATP and evolution of molecular oxygen. The NADPH2 and ATP are energy-rich, having caught the electrons that became available when light impinged upon chlorophyll. They form the assimilatory power, utilized for CO_2-fixation. The events of CO_2-fixation is light independent reaction and is designated as dark reaction.

LIGHT REACTION

Light reaction consists of two phase:

Phase I-Energy absorption (Absorption and retention of light by the photosynthetic pigments); and

Phase II-Energy transduction (conversion of light energy absorbed in phase I into chemical energy-ATP and $NADPH_2$ by photophosphorylation).

Phase I. Energy absorption.

Photosynthetic Units-The events of light reaction are mediated through photosynthetic units, a photosynthetic unit being the smallest group of pigment molecules, together with their lipo-protein associate substances, able to bring about a photochemical act (Photoact). The term,

photochemical act, means absorption and migration of a light quantum by a trapping centre, as a result of which an electron is released. Emerson and Arnold thought that a photosynthetic unit (PSU) contained at least 2500 chlorophyll molecules, but recent work by Bessel Kock indicates that a photosynthetic unit contains only about 250 chlorophyll molecules. The occurrence of PSU as a distinct morphological entity was obtained by Park and his co-workers and they christened it quantasome.

Absorption of light by pigments

All the light incident on photosynthesising surface is not used for photosynthesis. Much of it is lost and quite some is reflected back. Again, only a small fraction of the absorbed-light is used to drive photosynthesis. It is estimated that in full sunlight, just about 3% is used for photosynthetic purpose. Depending upon the pigment composition, the various plant groups absorb and utilise light of different spectral regions. Most green plants absorb light in the visible spectrum (390-700 nm), whereas purple bacteria employ wavelengths ranging from near ultraviolet to infrared (800-950 nm). This range of the spectrum through which photosynthesis can take place is called photosynthetically active radiation. But the entire range is not employable in photosynthesis. The green plants, for example, absorb light maximally in the red and blue regions of the spectrum. A study of the absorption spectra shows the quantitative relationship between the wavelength of light and its absorption by the pigment in question. Thus, we see that chlorophyll-a has its absorption peaks at 660 nm and 430 nm chlorophyll-b at 648 nm and 456 nm carotene at 478 nm and 449 nm and xanthophyll same as carotene.

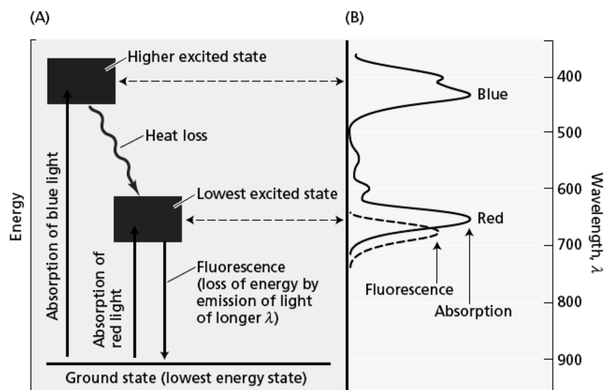


FIGURE Light absorption and emission by chlorophyll. (A) Energy level diagram. Absorption or emission of light is indicated by vertical lines that connect the ground state with excited electron states. The blue and red absorption bands of chlorophyll (which absorb blue and red photons, respectively) correspond to the upward vertical arrows, signifying that energy absorbed from light causes the molecule to change from the ground state to an excited state. The downward-pointing arrow indicates fluorescence, in which the molecule goes from the lowest excited state to the ground state while re-emitting energy as a photon. (B) Spectra of absorption and fluorescence. The long-wavelength (red) absorption band of chlorophyll corresponds to light that has the energy required to cause the transition from the ground state to the first excited state. The short-wavelength (blue) absorption band corresponds to a transition to a higher excited state.

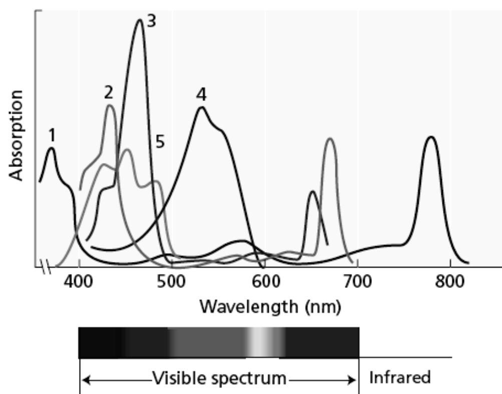


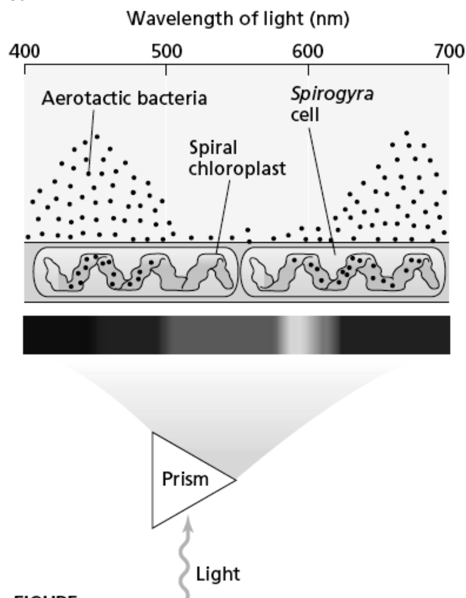
FIGURE Absorption spectra of some photosynthetic pigments. Curve 1, bacteriochlorophyll *a*; curve 2, chlorophyll *a*; curve 3, chlorophyll *b*; curve 4, phycoerythrobilin; curve 5, β-carotene. The absorption spectra shown are for pure pigments dissolved in nonpolar solvents, except for curve 4, which represents an aqueous buffer of phycoerythrin, a protein from cyanobacteria that contains a phycoerythrobilin chromophore covalently attached to the peptide chain. In many cases the spectra of photosynthetic pigments in vivo are substantially affected by the environment of the pigments in the photosynthetic membrane.

Light Trap: Chlorophyll-a utilises the light that it absorbs on its own and also the light transferred to it by other pigments. This funnelling of light from other pigments to chlorophyll a has been called light trap or light sink. The light trap makes for a much better light-harvesting efficiency, for it ensures funnelling of light quanta towards one acceptor molecule of chlorophyll.

The light reaction actually consists of two photochemical reactions which are separated both in time and space. They are designated as Photoact I and Photoact II and the two reactions are mediated by two different systems whose composition differ in terms of pigments, electron carriers and light trap mechanisms. The mediating agencies of the two photoacts are respectively called Photosystem I and Photosystem II.

Photosystems: The concept of two photosystems originated in the work of Emerson and Lewis (1943). Working on the action spectrum for the pigments of Chlorella, they found that at wavelengths of light between 600 and 680 nm (wavelengths corresponding to the 'red' region of the spectrum) the evolution of oxygen was at its maximum. But when light of wavelengths beyond 680 nm, a region of the spectrum referred to as 'far-red' was supplied there was a drop in the evolution of oxygen, indicative of lessened photosynthesis efficiency. This observation had been christened the red drop. His research group found that if light of shorter wave lengths was provided at the same time as the longer red wavelengths, photosynthesis was even

faster than when either of wavelengths alone was provided This synergism or enhancement became known as the Emerson enhancement effect. These two observations-the red drop effect and enhancement effect led to the first indication that the light reaction has two sites of action, one in the red region of the spectrum and the other in the far-red.



FIGURE

Schematic diagram of the action spectrum measurements by T. W. Engelmann. Engelmann projected a spectrum of light onto the spiral chloroplast of the filamentous green alga Spirogyra and observed that oxygen-seeking bacteria introduced into the system collected in the region of the spectrum where chlorophyll pigments absorb. This action spectrum gave the first indication of the effectiveness of light absorbed by accessory pigments in driving photosynthesis.

The explanation offered for these two effects is that the light reaction actually consists of two photoacts, photoact I and photoact II, mediated by two photosystems, photosystem I and photosystem II. Photosystem I is driven by the far-red light and when it operates alone, it produces the red drop effect. But when it operates along with photosystem II, which functions in the red region, enhancement effect is produced. Physical separation of the two photosystems had been sucessfully carried out and their functions clarified.

Photosystem I has been located in the thylakoid membranes. It is made up of three forms of chlorophyll-a, one absorbing maximally at 683 nm, the second absorbing maximally at 695 nm and the third at 670 nm. The last of these has been called P-700. Photosystem II had. been located in the stroma thylakoids. It is made up of two forms of chlorophyll-a with maximum absorption at 670 and 690 nm. The second form is christened P-690. Each photosystem has three components : (i) a reaction centre made up of a special chlorophyll molecule-in photosystem I it is a protein-bound chlorophyll-a molecule, P-700; in photosystem II it is P-690. The reaction centres are the actual sites where light energy is converted to chemical energy. (ii) some electron carriers-in photosystem I, X, plastocyanin, cytochrome-f and ferrodoxin as the electron carrier; photosystem II has plastoquinone and cytochrome b-559. (iii) other chlorophyll and carotenoids, which merely serve to transfer the light absorbed by them to the active centres.

Photosystem I takes part in both cyclic and non-cyclic photophosphorylations. PS-I can carryon cyclicphotosphosphorylation independently. Normally it drives an electron from photosystem II to $NADP^+$.

Photosystem II picks up electron released during photolysis of water. The same is extruded on absorption of light energy. As the extruded electron passes over cytochrome complex, sufficient energy is released to take part in the synthesis of ATP from ADP and inorganic phosphate. This photophosphorylation is noncyclic. PS II can operate only in conjunction with PS I.

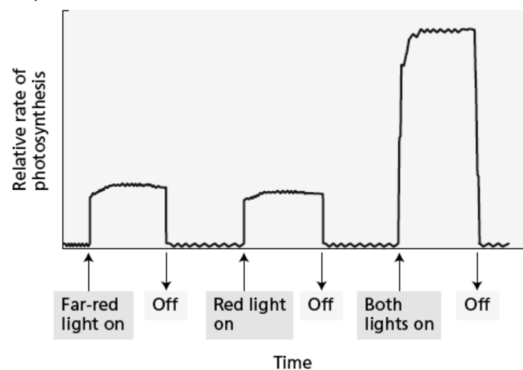


FIGURE Enhancement effect. The rate of photosynthesis when red and far-red light are given together is greater than the sum of the rates when they are given apart. The enhancement effect provided essential evidence in favor of the concept that photosynthesis is carried out by two photochemical systems working in tandem but with slightly different wavelength optima.

Phase II : Energy Transduction

The excited molecules of P-700. and P-690 transduce their energies to generate ATP and $NADPH_2$. Molecular oxygen is also produced but it escapes out of the photosynthetic system. ATP and $NADPH_2$ together constitute the assimilatory power and are employed in the fixation of CO_2 in the dark reaction.

Photophosphorylation

Photophosphorylation is the light driven or light energized synthesis of ATP. It was discovered by Amon et al in 1954. Photophosphorylation is of two main types, cyclic and noncyclic.

Cyclic Photophosphorylation: It is a process of photophosphorylation in which an electron expelled by the excited photocentre is returned to it after passing through a series of electron carriers. Cyclic photophosphorylation is performed by photosystem I only. Its photocentre P_{700} extrudes an electron with a gain of 23 kcal/mole of energy after absorbing a photon of light (hv). After losing the electron the photocentre becomes oxidised. The expelled electron passes through a series of carriers including X, ferredoxin, plastoquinone, cytochrome complex and plastocyanin before returning to photocentre.

While passing between ferredoxin and plastoquinone and/ or over the cytochrome complex, the electron loses sufficient energy to form ATP from ADP and inorganic phosphate.

Halobacteria or halophile bacteria also perform photophosphorylation but ATP thus produced is not used in synthesis of food. These bacteria possess purple pigment bacteriorhodopsin attached to plasma membrane. As light falls on the pigment, it creates a proton pump which is used in ATP synthesis.

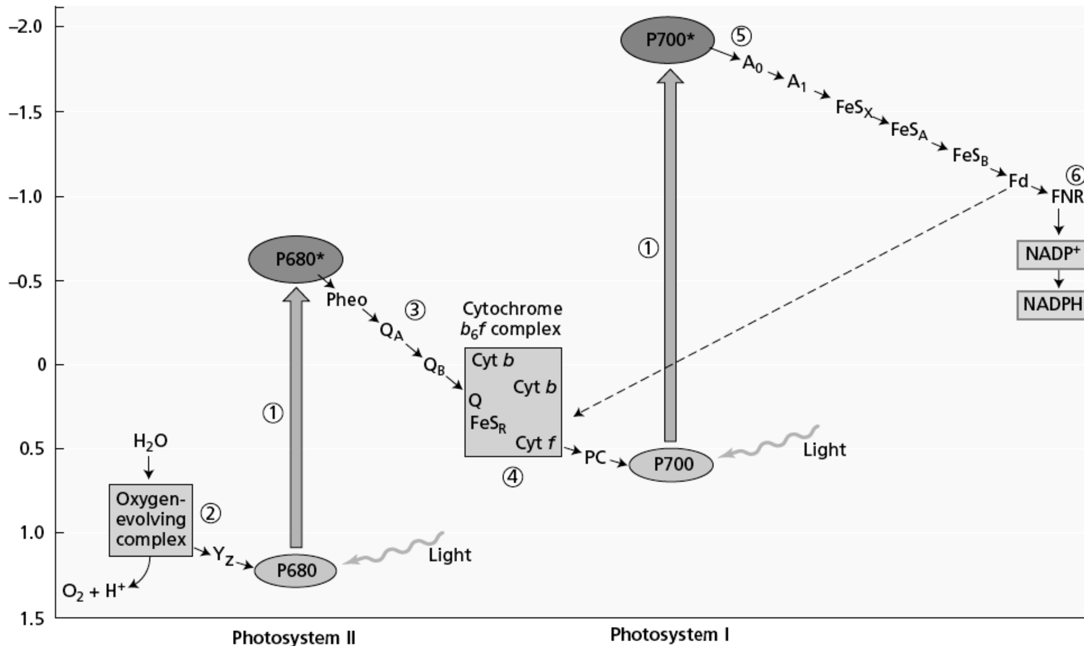


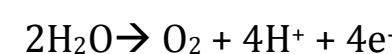
FIGURE Detailed Z scheme for O_2-evolving photosynthetic organisms. The redox carriers are placed at their midpoint redox potentials (at pH 7). (1) The vertical arrows represent photon absorption by the reaction center chlorophylls: P680 for photosystem II (PSII) and P700 for photosystem I (PSI). The excited PSII reaction center chlorophyll, P680*, transfers an electron to pheophytin (Pheo). (2) On the oxidizing side of PSII (to the left of the arrow joining P680 with P680*), P680 oxidized by light is re-reduced by Y_z, that has received electrons from oxidation of water. (3) On the reducing side of PSII (to the right of the arrow joining P680 with P680*), pheophytin transfers electrons to the acceptors Q_A and Q_B, which are plastoquinones. (4) The cytochrome b_6f complex transfers electrons to plastocyanin (PC), a soluble protein, which in turn reduces $P700^+$ (oxidized P700). (5) The acceptor of electrons from P700* (A_0) is thought to be a chlorophyll, and the next acceptor (A_1) is a quinone. A series of membrane-bound iron–sulfur proteins (FeS_X, FeS_A, and FeS_B) transfers electrons to soluble ferredoxin (Fd). (6) The soluble flavoprotein ferredoxin–NADP reductase (FNR) reduces $NADP^+$ to NADPH, which is used in the Calvin cycle to reduce CO_2 . The dashed line indicates cyclic electron flow around PSI.

Noncyclic Photophosphorylation: It is the normal process of photophosphorylation in which the electron expelled by the excited photocentre does not return to it. Non-cyclic photophosphorylation is carried out in collaboration of both photosystems I and II. Electron released during photolysis of water is picked up by photocentre of PS II called P 680

The same is extruded out when the photocentre absorbs light energy (*hv*). The extruded electron has an energy equivalent to 23 kcal/mole. It passes through a series of electron carriers Q, PQ, cytochrome complex and plastocyanin. While passing over cytochrome complex, the electron loses sufficient energy for the syntheisis of ATP. The electron is handed over to photocentre P 700 of PSI by plastocyanin P 700 extrudes the electron after absorbing light energy. The extruded electron passes through X, Fe-S centre A (ferredoxin), and NADP-reductase which combines it with NADP+ for becoming reduced H^+ released during photolysis to form NADPH. ATP synthesis is not direct. The energy released by electron is actually used for pumping H+ ions across the thylakoid membrane. It creates a proton gradient. The gradient triggers the coupling factor to syntl1esize ATP from ADP and inorganic phosphate.

Oxygen evolution:

The oxygen that is evolved during photosynthesis comes from water and it is part of the photoact II mediated by PSII. The light energy trapped by this system excites P-690 and two electrons are ejected. The energy is used to remove two electrons from the hydrogen of the water and boost them to a higher energy level. At this point, the molecular oxygen which escapes from the photosynthetic system is formed. The electrons pass through the carriers plastoquinone (PQ), cytochrome b-559, cytochrome-F, plastocyanin and finally end up in P-700 to take it back to the ground state. Thus in photosystem II, the electrons that brings the excited chlorophyll molecule to the ground state comes from photolysis of water. Another aspect of oxygen evolution during photosynthesis is its relationship to the presence of certain ions in the medium such as Cl^-, Mn2+ and bicarbonate.



Both the photosystems working in union produce, for every two turns, two molecules of $NADPH_2$, three ATPs, and a molecule of oxygen from two water molecules.

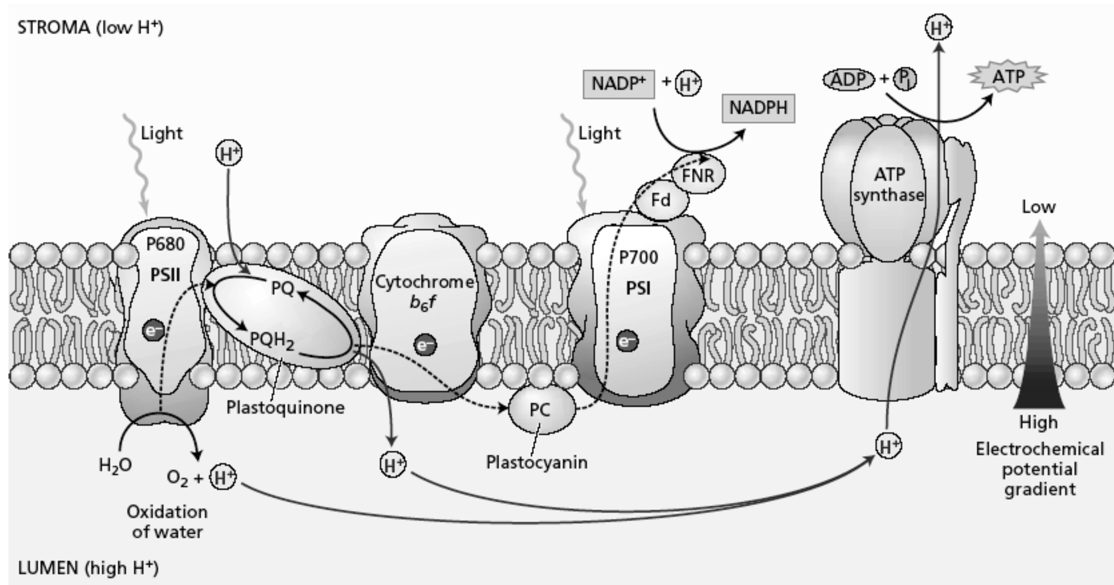


FIGURE The transfer of electrons and protons in the thylakoid membrane is carried out vectorially by four protein complexes. Water is oxidized and protons are released in the lumen by PSII. PSI reduces $NADP^+$ to NADPH in the stroma, via the action of ferredoxin (Fd) and the flavoprotein ferredoxin–NADP reductase (FNR). Protons are also transported into the lumen by the action of the cytochrome b_6f complex and contribute to the electrochemical proton gradient. These protons must then diffuse to the ATP synthase enzyme, where their diffusion down the electrochemical potential gradient is used to synthesize ATP in the stroma. Reduced plastoquinone (PQH_2) and plastocyanin transfer electrons to cytochrome b_6f and to PSI, respectively. Dashed lines represent electron transfer; solid lines represent proton movement.

Some Herbicides Block Electron Flow

The use of herbicides to kill unwanted plants is widespread in modern agriculture. Many different classes of herbicides have been developed, and they act by blocking amino acid, carotenoid, or lipid biosynthesis or by disrupting cell division. Other herbicides, such as DCMU (dichlorophenyldimethylurea) and paraquat, block photosynthetic electron flow (Figure). DCMU is also known as diuron. Paraquat has acquired public notoriety because of its use on marijuana crops.

Many herbicides, DCMU among them, act by blocking electron flow at the quinone acceptors of photosystem II, by competing for the binding site of plastoquinone that is normally occupied by QB. Other herbicides, such as paraquat, act by accepting electrons from the early acceptors of photosystem I and then reacting with oxygen to form superoxide, O_2^- , a species that is very damaging to chloroplast components, especially lipids.

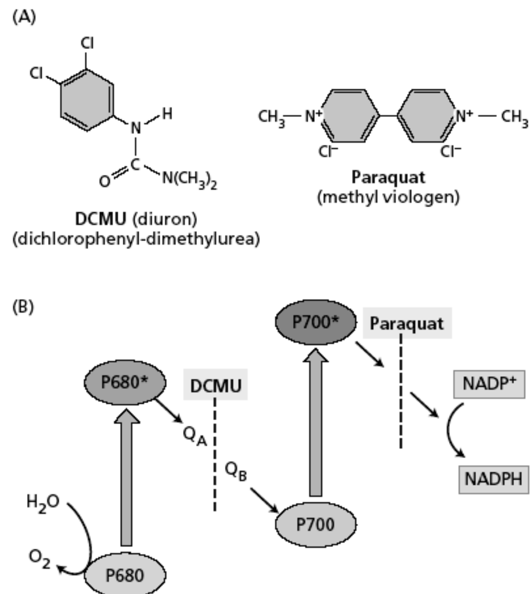


FIGURE Chemical structure and mechanism of action of two important herbicides. (A) Chemical structure of dichlorophenyl-dimethylurea (DCMU) and methyl viologen (paraquat), two herbicides that block photosynthetic electron flow. DCMU is also known as diuron. (B) Sites of action of the two herbicides. DCMU blocks electron flow at the quinone acceptors of photosystem II, by competing for the binding site of plastoquinone. Paraquat acts by accepting electrons from the early acceptors of photosystem I.

Carotenoids Serve as Photoprotective Agents

In addition to their role as accessory pigments, carotenoids play an essential role in **photoprotection**. The photosynthetic membrane can easily be damaged by the large amounts of energy absorbed by the pigments if this energy cannot be stored by photochemistry; this is why a protection mechanism is needed. The photoprotection mechanism can be thought of as a safety valve, venting excess energy before it can damage the organism. When the energy stored in chlorophylls in the excited state is rapidly dissipated by excitation transfer or photochemistry, the excited state is said to be **quenched**.

If the excited state of chlorophyll is not rapidly quenched by excitation transfer or photochemistry, it can react with molecular oxygen to form an excited state of oxygen known as **singlet oxygen** ($^1O_2$*). The extremely reactive singlet oxygen goes on to react with and damage many cellular components, especially lipids. Carotenoids exert their photoprotective action by rapidly quenching the excited state of chlorophyll. The excited state of carotenoids does not have sufficient energy to form singlet oxygen, so it decays back to its ground state while losing its energy as heat.

Mutant organisms that lack carotenoids cannot live in the presence of both light and molecular oxygen—a rather difficult situation for an O2-evolving photosynthetic organism.

For non-O_2-evolving photosynthetic bacteria, mutants that lack carotenoids can be maintained under laboratory conditions if oxygen is excluded from the growth medium.

Recently carotenoids were found to play a role in nonphotochemical quenching, which is a second protective and regulatory mechanism.

Some Xanthophylls Also Participate in Energy Dissipation

Nonphotochemical quenching, a major process regulating the delivery of excitation energy to the reaction center, can be thought of as a "volume knob" that adjusts the flow of excitations to the PSII reaction center to a manageable level, depending on the light intensity and other conditions. The process appears to be an essential part of the regulation of antenna systems in most algae and plants.

Nonphotochemical quenching is the quenching of chlorophyll fluorescence by processes other than photochemistry. As a result of nonphotochemical quenching, a large fraction of the excitations in the antenna system caused by intense illumination are quenched by conversion into heat. Nonphotochemical quenching is thought to be involved in protecting the photosynthetic machinery against overexcitation and subsequent damage.

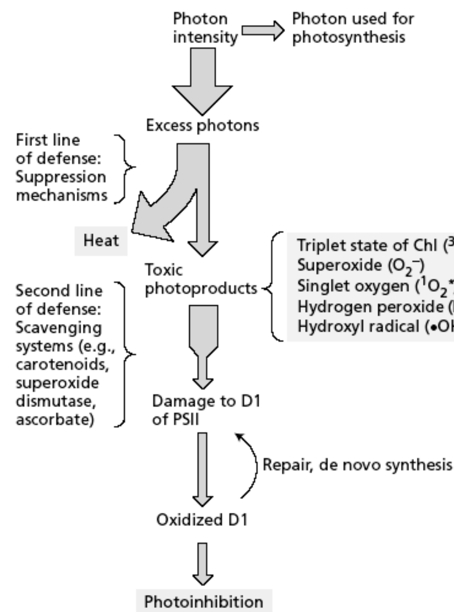
The molecular mechanism of nonphotochemical quenching is not well understood, although it is clear that the pH of the thylakoid lumen and the state of aggregation of the antenna complexes are important factors. Three carotenoids, called **xanthophylls**, are involved in nonphotochemical quenching: violaxanthin, antheraxanthin, and zeaxanthin.

In high light, violaxanthin is converted into zeaxanthin, via the intermediate antheraxanthin, by the enzyme violaxanthin de-epoxidase. When light intensity decreases, the process is reversed. Binding of protons and zeaxanthin to light-harvesting antenna proteins is thought to cause conformational changes that lead to quenching and heat dissipation. Nonphotochemical quenching appears to be preferentially associated with a peripheral antenna complex of photosystem II, the PsbS protein.

The Photosystem II Reaction Center Is Easily Damaged

Another effect that appears to be a major factor in the stability of the photosynthetic apparatus is photoinhibition, which occurs when excess excitation arriving at the PSII reaction center leads to its inactivation and damage. **Photoinhibition** is a complex set of molecular processes, defined as the inhibition of photosynthesis by excess light.

Photoinhibition is reversible in early stages. Prolongued inhibition, however, results in damage to the system such that the PSII reaction center must be disassembled and repaired. The main target of this damage is the D1 protein that makes up part of the PSII reaction center complex (see Figure). When D1 is damaged by excess light, it must be removed from the membrane and replaced with a newly synthesized molecule. The other components of the PSII reaction center are not damaged by excess excitation and are thought to be recycled, so the D1 protein is the only component that needs to be synthesized.



Photosystem I Is Protected from Active Oxygen Species

Photosystem I is particularly vulnerable to damage from active oxygen species. The ferredoxin acceptor of PSI is a very strong reductant that can easily reduce molecular oxygen to form superoxide (O_2^-). This reduction competes with the normal channeling of electrons to the reduction of $NADP^+$ and other processes. Superoxide is one of a series of active oxygen species that can be very damaging to biological membranes.

Superoxide formed in this way can be eliminated by the action of a series of enzymes, including superoxide dismutase and ascorbate peroxidase.

Thylakoid Stacking Permits Energy Partitioning between the Photosystems

The fact that photosynthesis in higher plants is driven by two photosystems with different light-absorbing properties poses a special problem. If the rate of delivery of energy to PSI and PSII is not precisely matched and conditions are such that the rate of photosynthesis is limited by the available light (low light intensity), the rate of electron flow will be limited by the photosystem that is receiving less energy.

In the most efficient situation, the input of energy would be the same to both photosystems. However, no single arrangement of pigments would satisfy this requirement

because at different times of day the light intensity and spectral distribution tend to favor one photosystem or the other.

This problem can be solved by a mechanism that shifts energy from one photosystem to the other in response to different conditions. Such a regulating mechanism has been shown to operate in different experimental conditions. The observation that the overall quantum yield of photosynthesis is nearly independent of wavelength strongly suggests that such a mechanism exists.

Thylakoid membranes contain a protein kinase that can phosphorylate a specific threonine residue on the surface of LHCII, one of the membrane-bound antenna pigment proteins described earlier in the chapter. When LHCII is not phosphorylated, it delivers more energy to photosystem II, and when it is phosphorylated, it delivers more energy to photosystem I.

The kinase is activated when plastoquinone, one of the electron carriers between PSI and PSII, accumulates in the reduced state. Reduced plastoquinone accumulates when PSII is being activated more frequently than PSI. The phosphorylated LHCII then migrates out of the stacked regions of the membrane into the unstacked regions, probably because of repulsive interactions with negative charges on adjacent membranes. The lateral migration of LHCII shifts the energy balance toward photosystem I, which is located in the stroma lamellae, and away from photosystem II, which is located in the stacked membranes of the grana. This situation is called *state 2*. If plastoquinone becomes more oxidized because of excess excitation of photosystem I, the kinase is deactivated and the level of phosphorylation of LHCII is decreased by the action of a membrane-bound phosphatase.

LHCII then moves back to the grana, and the system is in *state 1*. The net result is a very precise control of the energy distribution between the photosystems, allowing the most efficient use of the available energy.

BLACKMAN'S REACTION

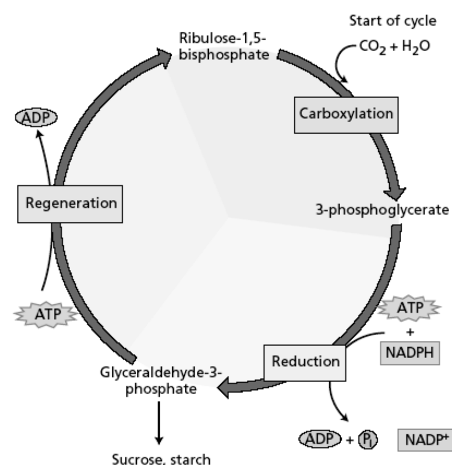


FIGURE The Calvin cycle proceeds in three stages: (1) carboxylation, during which CO_2 is covalently linked to a carbon skeleton; (2) reduction, during which carbohydrate is formed at the expense of the photochemically derived ATP and reducing equivalents in the form of NADPH; and (3) regeneration, during which the CO_2 acceptor ribulose-1,5-bisphosphate re-forms.

Phase III: Energy Stabilization

The production of carbon dioxide to a carbohydrate is the essence of this phase and this is accomplished through the employment of the assimilatory power (ATP and $NADPH_2$) generated in the light reaction. This energy stabilization is a dark reaction. It does not require light, instead assimilatory power (ATP and $NADPH_2$ produced during photochemical phase is used here in fixation and reduction of CO_2. The enzymes required for the process are present in the matrix or stroma of the chloroplast. There are two mam pathways for the biosynthetic or dark phase-Calvin cycle and C_4 dicarboxylic acid cycle. The plants exhibiting the two are respectively called C_3 and C_4 plants.

Calvin cycle (the photosynthetic carbon reduction cycle or the C-3 photosynthetic pathway):

This cycle was discovered by Calvin, Benson and their colleagues using unicellular algae Chlorella pyrenoidosa and Scenedesmus obliques and radioactive isotope of ^{14}C with a half-life of more than 5000 years.

Phases of Calvin Cycle: Calvin cycle is divided into the following three phases-carboxylation, glycolytic reversal and regeneration of RuBP.

1. Carboxylation: It requires ribulose-1, 5-biphosphate or RuBP as acceptor of carbon dioxide and RuBP carboxylase or rubisco as enzyme. The enzyme was previously called carboxydismutase. Carbon dioxide combines with ribulose-1, 5-biphosphate to produce a transient intermediate compound called 2-carboxy 3-keto 1,5-biphosphoribotol. The intermediate splits up immediately in the presence of water to form the two molecules of 3-phosphoglyceric acid or PGA. It is the first stable product of photosynthesis.

2. Glycolytic Reversal: The processes involved in this step or phase are reversal of processes found during glycolysis part of respiration. Phosphoglyceric acid or PGA is further phosphorylated by ATP with the help of enzyme triose phosphate kinase. It give rise to 1, 3-diphosphoglyceric acid.

Diphosphoglyceric acid is reduced by NADPH through the agency of enzyme triose phosphate dehydrogenase. It produces glyceraldehyde 3-phosphate or 3-phosphoglyceraldehyde.

Glyceraldehyde-3 phosphate is a key product which is used in synthesis of both carbohydrates and fats. For forming carbohydrates, say glucose, a part of it is changed into its isomer called dihydroxyacetone-3-phosphate. The enzyme that catalyses the reaction is phosphate isomerase.

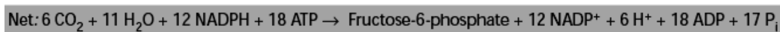
The two isomers condense in the presence of enzyme aldolase forming fructose 1, 6diphosphate.

Fructose 1,6-diphosphate (FOP) loses one phosphate group, forms fructose 6-phosphate (F 6-P) which is then changed to glucose-6-phosphate (G 6 P). The latter can produce glucose or become part of sucrose and polysaccharide.
As glucose is a six carbon compound, six turns of Calvin cycle are required to synthesise its one molecule.

3. Regeneration of RuBP: Fructose 6-phosphate (F 6-P) and glyceraldehyde 3-phosphate (GAP) react to form erythrose 4-phosphate (E 4-P) and xylulose 5-phosphate (X 5-P). Erythrose 4-phosphate combines with dihydroxy acetone 3-phosphate to produce sedoheptulose 1 : 7-diphosphate (SDP) which loses a molecule of phosphate and

gives rise to sedoheptulose 7-phosphate (S 7-P). Sedoheptulose 7-phosphate reacts with glyceraldehyde 3-phosphate to produce xylulose 5-phosphate (X 5-P) and ribose 5-phosphate. (R 5-P). Both of these are changed to their isomer ribulose 5-phosphate (Ru 5-P). Ribulose 5-phosphate picks up a second phosphate from ATP to become changed into ribulose 1, 5 biphosphate (RuBP).

FIGURE The Calvin cycle. The carboxylation of three molecules of ribulose-1,5- bisphosphate leads to the *net* synthesis of one molecule of glyceraldehyde-3-phos phate and the regeneration of the three molecules of starting material. This process starts and ends with three molecules of ribulose-1,5-bisphosphate, reflecting the cyclic nature of the pathway.



REGULATION OF THE CALVIN CYCLE

The high energy efficiency of the Calvin cycle indicates that some form of regulation ensures that all intermediates in the cycle are present at adequate concentrations and that the cycle is turned off when it is not needed in the dark. In general, variation in the concentration or in the specific activity of enzymes modulates catalytic rates, thereby adjusting the level of metabolites in the cycle.

Changes in gene expression and protein biosynthesis regulate enzyme concentration. Posttranslational modification of proteins contributes to the regulation of enzyme activity. At the genetic level the amount of each enzyme present in the chloroplast stroma is regulated by mechanisms that control expression of the nuclear and chloroplast genomes.

Short-term regulation of the Calvin cycle is achieved by several mechanisms that optimize the concentration of intermediates. These mechanisms minimize reactions operating in opposing directions, which would waste resources. Two general mechanisms can change the kinetic properties of enzymes:
1. The transformation of covalent bonds such as the reduction of disulfides and the carbamylation of amino groups, which generate a chemically modified enzyme.
2. The modification of noncovalent interactions, such as the binding of metabolites or changes in the composition of the cellular milieu (e.g., pH). In addition, the binding of the enzymes to the thylakoid membranes enhances the efficiency of the Calvin cycle, thereby achieving a higher level of organization that favors the channeling and protection of substrates.

Calvin cycle is not a Dark Reaction: Light-Dependent Enzyme Activation Regulates the Calvin Cycle

Five light-regulated enzymes operate in the Calvin cycle:

1. Rubisco
2. NADP:glyceraldehyde-3-phosphate dehydrogenase
3. Fructose-1,6-bisphosphatase
4. Sedoheptulose-1,7-bisphosphatase
5. Ribulose-5-phosphate kinase

The last four enzymes contain one or more disulfide (—S—S—) groups. Light controls the activity of these four enzymes via the **ferredoxin–thioredoxin system**, a covalent thiol-based oxidation–reduction mechanism identified by Bob Buchanan and colleagues. In the dark these residues exist in the oxidized state (—S—S—), which renders the enzyme inactive or subactive. In the light the —S—S— group is reduced to the sulfhydryl state (—SH HS—). This redox change leads to activation of the enzyme. The resolution of the crystal structure of each member of the ferredoxin– thioredoxin system and of the target enzymes fructose-1,6-bisphosphatase and NADP : malate dehydrogenase have provided valuable information about the mechanisms involved.

This sulfhydryl (also called dithiol) signal of the regulatory protein thioredoxin is transmitted to specific target enzymes, resulting in their activation In some cases (such as fructose-1,6-bisphosphatase), the thioredoxin-linked activation is enhanced by an effector (e.g., fructose-1,6-bisphosphate substrate). Inactivation of the target enzymes observed upon darkening appears to take place by a reversal of the reduction (activation) pathway. That is, oxygen converts the thioredoxin and target enzyme from the reduced state (—SH HS—) to the oxidized state (—S—S—) and, in so doing, leads to inactivation of the enzyme. The last four of the enzymes listed here are regulated directly by thioredoxin; the first, rubisco, is regulated indirectly by a thioredoxin accessory enzyme, rubisco activase.

C2 cycle: Photorespiration (Respiration Associated with Photosynthetic Tissues)

It was discovered by Decker and Tio in 1959. Photorespiration is the light dependent utilization of oxygen and release of carbon dioxide by the photosynthetic organs of a plant. Normally photosynthetic organs do the reverse in the light i.e., uptake of CO_2 and release of O_2. Therefore, photorespiration is difficult to demonstrate. It is inferred from (i) Decrease in the rate of net photosynthesis when oxygen concentration is increased from 2-3% to 21% (ii) Sudden increased evolution of O_2 when an illuminated green organ is transferred to dark.

The site for photorespiration is chloroplast. Peroxisome is required for completing the process. RuBP carboxylase is changed to RuBP oxygenase. This happens at high temperature and high oxygen concentration. At high temperature and high oxygen concentration, the affinity of RuBP carboxylase for CO_2 decreases and the affinity for O_2 increases. High temperature occurs in tropical areas. Therefore, tropical plants are the major sufferers. At high temperature, RuBP carboxylase functions as oxygenase and instead of fixing carbon dioxide, oxidises ribulose 1, 5-biphosphate to produce phosphoglyceric acid and phosphoglycolate.

Phosphoglycolate is hydrolysed to form glycolate. Glycolate usually passes into peroxisome of the mesophyll cell and forms glyoxylate. Glyoxylate is aminated and gives rise to amino acid glycine. Inside mitochondrion and even cytoplasm the two molecules of glycine condense to form a molecule of serine, $C0_2$ and ammonia are released in the process. Serine can further be deaminated to form PGA. The latter passes into chloroplasts for synthesis of pho-tosynthetic products as well as photorespiration. Since photorespiration involves the synthesis of two-carbon compounds, it is also called C_2 cycle.

Photorespiration does not produce energy or reducing power. Rather, it consumes energy. Further, it undoes the work of photosynthesis. It may reduce photosynthesis upto 50%. Therefore, photorespiration is a highly wasteful process. This happens only in case of C3 plants. C4 plants have overcome the problem of photorespiration by performing Calvin Cycle in the interior of leaves (bundle sheath cells) where both temperature and oxygen are lower. They have further ensured high CO_2 supply to cells performing Calvin cycle.

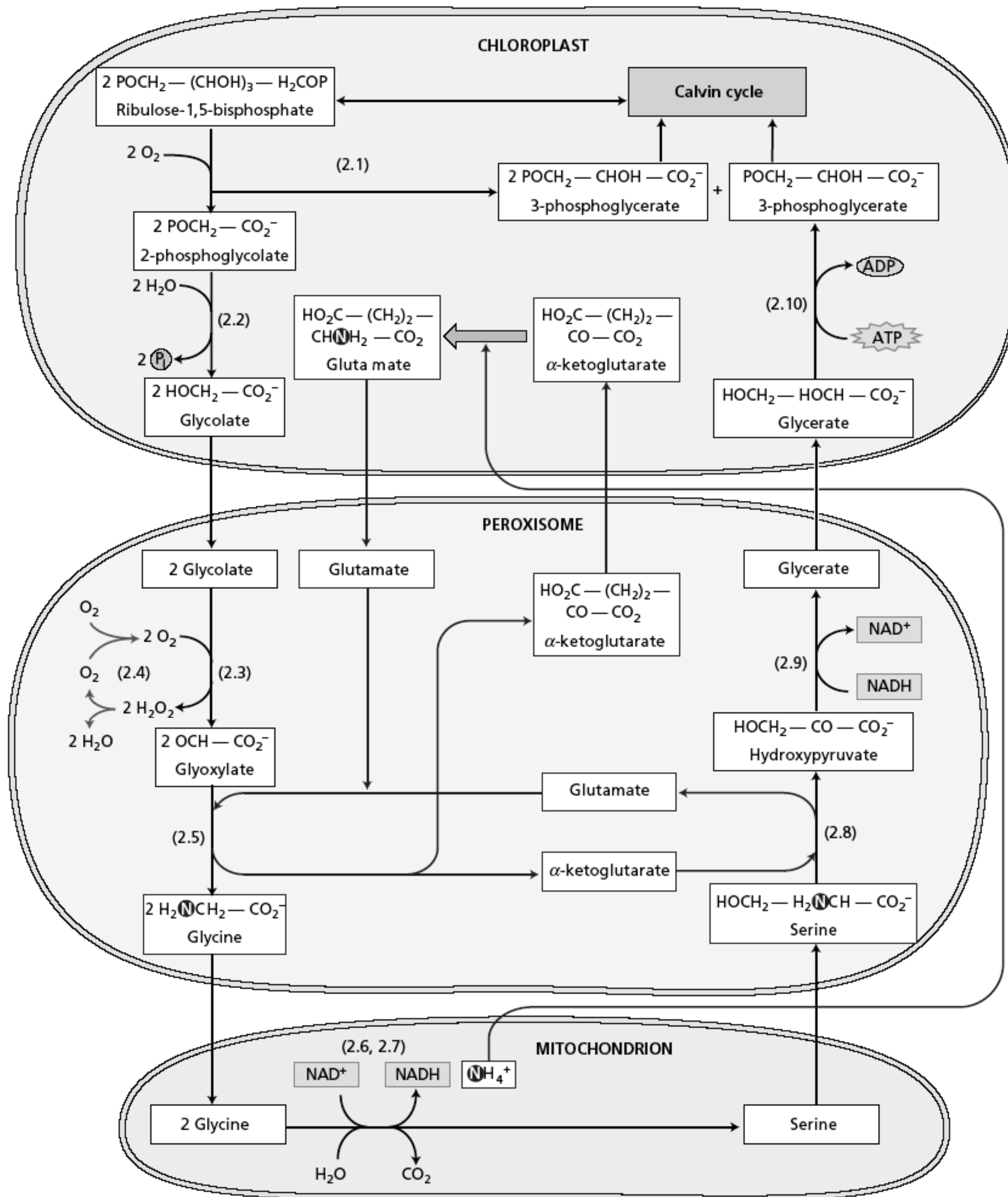


FIGURE The main reactions of the photorespiratory cycle. Operation of the C_2 oxidative photosynthetic cycle involves the cooperative interaction among three organelles: chloroplasts, mitochondria, and peroxisomes. Two molecules of glycolate (four carbons) transported from the chloroplast into the peroxisome are converted to glycine, which in turn is exported to the mitochondrion and transformed to serine (three carbons) with the concurrent release of carbon dioxide (one carbon). Serine is transported to the peroxisome and transformed to glycerate. The latter flows to the chloroplast where it is phosphorylated to 3-phosphoglycerate and incorporated into the Calvin cycle. Inorganic nitrogen (ammonia) released by the mitochondrion is captured by the chloroplast for the incorporation into amino acids by using appropiate skeletons (α-ketoglutarate). The heavy arrow in red marks the assimilation of ammonia into glutamate catalyzed by glutamine synthetase. In addition, the uptake of oxygen in the peroxisome supports a short oxygen cycle coupled to oxidative reactions. The flow of carbon, nitrogen and oxygen are indicated in black, red and blue, respectively. See Table for a description of each numbered reaction.

TABLE Reactions of the C_2 oxidative photosynthetic carbon cycle

Enzyme	Reaction
1. Ribulose-1,5-bisphosphate carboxylase/oxygenase (chloroplast)	2 Ribulose-1,5-bisphosphate + 2 O_2 → 2 phosphoglycolate + 2 3-phosphoglycerate + 4 H^+
2. Phosphoglycolate phosphatase (chloroplast)	2 Phosphoglycolate + 2 H_2O → 2 glycolate + 2 P_i
3. Glycolate oxidase (peroxisome)	2 Glycolate + 2 O_2 → 2 glyoxylate + 2 H_2O_2
4. Catalase (peroxisome)	2 H_2O_2 → 2 H_2O + O_2
5. Glyoxylate:glutamate aminotransferase (peroxisome)	2 Glyoxylate + 2 glutamate → 2 glycine + 2 α-ketoglutarate
6. Glycine decarboxylase (mitochondrion)	Glycine + NAD^+ + H^+ + H_4-folate → NADH + CO_2 + NH_4^+ + methylene-H_4-folate
7. Serine hydroxymethyltransferase (mitochondrion)	Methylene-H_4-folate + H_2O + glycine → serine + H_4-folate
8. Serine aminotransferase (peroxisome)	Serine + α-ketoglutarate → hydroxypyruvate + glutamate
9. Hydroxypyruvate reductase (peroxisome)	Hydroxypyruvate + NADH + H^+ → glycerate + NAD^+
10. Glycerate kinase (chloroplast)	Glycerate + ATP → 3-phosphoglycerate + ADP + H^+

Note: Upon the release of glycolate from the chloroplast (reactions 2 → 3), the interplay of this organelle with the peroxisome and the mitochondrion drives the following overall reaction:

$$2\ \text{Glycolate} + \text{glutamate} + O_2 \rightarrow \text{glycerate} + \alpha\text{-ketoglutarate} + NH_4^+ + CO_2 + H_2O$$

The 3-phosphoglycerate formed in the chloroplast (reaction 10) is converted to ribulose-1,5-bisphosphate via the reductive and regenerative reactions of the Calvin cycle. The ammonia and α-ketoglutarate are converted to glutamate in the chloroplast by ferrodoxin-linked glutamate synthase (GOGAT).

P_i stands for inorganic phosphate.

The Biological Function of Photorespiration Is Unknown

Although the C2 oxidative photosynthetic carbon cycle recovers 75% of the carbon originally lost from the Calvin cycle as 2-phosphoglycolate, why does 2-phosphoglycolate form at all? One possible explanation is that the formation of 2-phosphoglycolate is a consequence of the chemistry of the carboxylation reaction, which requires an intermediate that can react with both CO_2 and O_2.

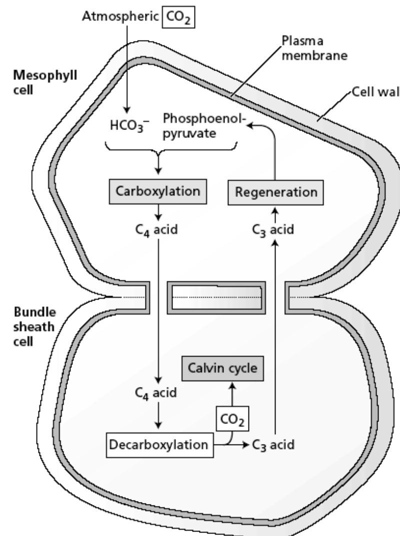
Such a reaction would have had little consequence in early evolutionary times if the ratio of CO_2 to O_2 in air were higher than it is today. However, the low CO_2:O_2 ratios prevalent in modern times are conducive to photorespiration, with no other function than the recovery of some of the carbon present in 2-phosphoglycolate.

Another possible explanation is that photorespiration is important, especially under conditions of high light intensity and low intercellular CO2 concentration (e.g., when stomata are closed because of water stress), to dissipate excess ATP and reducing power from the light reactions, thus preventing damage to the photosynthetic apparatus.

Arabidopsis mutants that are unable to photorespire grow normally under 2% CO_2, but they die rapidly if transferred to normal air. There is evidence from work with transgenic plants that photorespiration protects C3 plants from photooxidation and photoinhibition. Further work is needed to improve our understanding of the function of photorespiration.

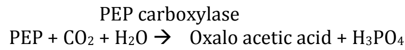
C4-DICARBOXYLIC ACID PATHWAY (HATCH SLACK PATHWAY, C4 PATHWAY)

It was worked out by Hatch and Slack (1965, 1967). Koitschak et al (1965) found that labelled carbon dioxide ($^{14}CO_2$) assimilated by Sugarcane leaves first appeared in a 4-carbon compound oxalo-acetic acid (OAA or oxaloacetate). Hatch and Slack found it a regular mode of CO_2-fixation in a number of tropical plants, both monocots and dicots, eg., Maize, Sugarcane, Sorghum, Panicum, Pennisetum, Atriplex, Amaranthus, Salsola etc. These plants are called **C4 plants** because of the first stable photosynthetic product being a 4-carbon-compound. Other plants are C_3 plants. C_4 plants often live in hot, arid and saline habitats. They have Kranz anatomy. In Kranz anatomy, the mesophyll is undifferentiated and its cells occur in concentric layers around vascular bundles having large bundle sheath cells. The mesophyll and bundle sheath cells are connected by plasmodesmata or cytoplasmic bridges. The chloroplasts of the mesophyll cells are smaller. They have well developed grana and a peripheral reticulum but no starch. The chloroplasts of the bundle sheath cells are larger. They have ill defined grana, a peripheral reticulum and starch grains.



In C4 plants, initial fixation of carbon dioxide occurs in mesophyll cells. The primary acceptor of C02 is phosphoenol pyruvate or PEP.

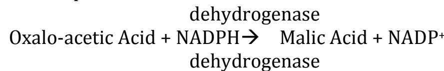
It combines with carbon dioxide in the presence of PEP carboxylase or pepco to form oxalo-acetic acid or oxaloacetate.



PEP carboxylase

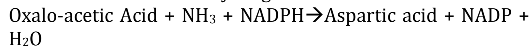
PEP + CO_2 + H_2O → Oxalo acetic acid + H_3PO_4

Oxalo-acetic acid is reduced to malic acid or aminated to form aspartic acid



dehydrogenase

Oxalo-acetic Acid + NADPH→ Malic Acid + $NADP^+$

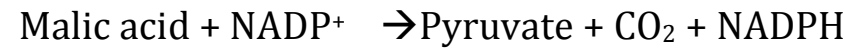


dehydrogenase

Oxalo-acetic Acid + NH_3 + NADPH→Aspartic acid + NADP + H_2O

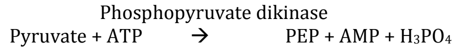
Malic acid or aspartic acid is translocated to bundle sheath cells through plasmodesmata. Inside the bundle sheath cells they are decarboxylated (and demainated in case of aspartic acid) to form pyruvate and CO_2.

Malic enzyme



Malic acid + $NADP^+$ →Pyruvate + CO_2 + NADPH

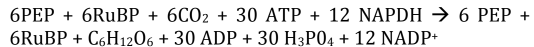
CO_2 is again fixed inside the bundle sheath cells through Calvin cycle. RuBP of Calvin cycle is called secondary or final acceptor of CO_2 in C4 plants. Pyruvate is sent back to mesophyll cells. Here, it is changed to phosphoenol pyruvate. Energy is required for this. The same is provided by ATP. The latter is changed into AMP (adenosine monophosphate).



Phosphopyruvate dikinase

Pyruvate + ATP → PEP + AMP + H_3PO_4

Conversion of AMP to ATP requires double the energy than conversion of ADP to ATP. Therefore, actual requirement of energy is equal to two molecules of ATP. This energy is in addition to 3 ATP required for fixation of one molecule of CO_2 through Calvin cycle. Therefore, C4 plants consume 5 ATP molecules per molecule of CO_2 fixed instead of 3 ATP molecules for C3 plants. For the formation of a glucose molecule, C4 plants require 30 ATP while C3 plants utilize only 18 ATP.



6PEP + 6RuBP + $6CO_2$ + 30 ATP + 12 NAPDH → 6 PEP + 6RuBP + $C_6H_{12}O_6$ + 30 ADP + 30 H_3P0_4 + 12 $NADP^+$

Reactions of the C_4 photosynthetic carbon cycle

Enzyme	Reaction
1. Phosphoenolpyruvate (PEP) carboxylase	Phosphoenolpyruvate + HCO_3^- → oxaloacetate + P_i
2. NADP:malate dehydrogenase	Oxaloacetate + NADPH + H^+ → malate + $NADP^+$
3. Aspartate aminotransferase	Oxaloacetate + glutamate → aspartate + α-ketoglutarate
4. NAD(P) malic enzyme	Malate + $NAD(P)^+$ → pyruvate + CO_2 + NAD(P)H + H^+
5. Phosphoenolpyruvate carboxykinase	Oxaloacetate + ATP → phosphoenolpyruvate + CO_2 + ADP
6. Alanine aminotransferase	Pyruvate + glutamate ↔ alanine + α-ketoglutarate
7. Adenylate kinase	AMP + ATP → 2 ADP
8. Pyruvate- orthophosphate dikinase	Pyruvate + P_i + ATP → phosphoenolpyruvate + AMP + PP_i
9. Pyrophosphatase	PP_i + H_2O → 2 P_i

Note: P_i and PP_i stand for inorganic phosphate and pyrophosphate, respectively.

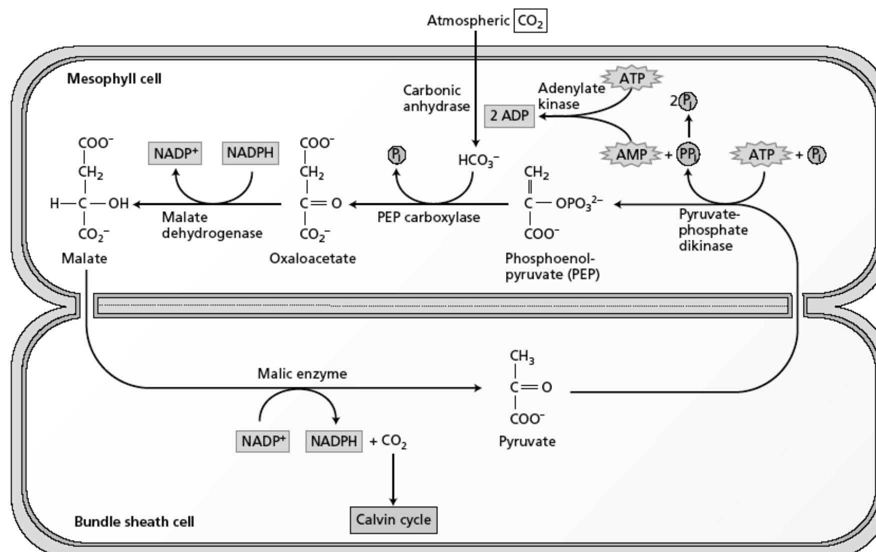


FIGURE The C_4 photosynthetic pathway. The hydrolysis of two ATP drives the cycle in the direction of the arrows, thus pumping CO_2 from the atmosphere to the Calvin cycle of the chloroplasts from bundle sheath cells.

Importance

1. The C4-plants are considered to possess greater photosynthetic efficiency, for they can utilize CO_2 until a level of 5 ppm is reached but the Calvin cycle plants cannot utilise CO_2 if the level falls below 40-50 ppm.

2. C4-plants can utilize greater light intensities and their temperature optima for photosynthesis exceeds those of C3 plants.

3. The chloroplasts of these plants seem to generate more ATP which of course makes for improved cellular work.

4. The presence of extensive peripheral reticulum in the chloroplasts of these plants indirectly suggests quicker transport of products and therefore greater utilisation of light and CO_2.

Energetics of the C_4 photosynthetic carbon cycle

Reactants		Products
Phosphoenolpyruvate + H_2O + NADPH + CO_2 (mesophyll)	→	malate + $NADP^+$ + P_i (mesophyll)
Malate + $NADP^+$	→	pyruvate + NADPH + CO_2 (bundle sheath)
Pyruvate + P_i + ATP	→	phosphoenolpyruvate + AMP + PP_i (mesophyll)
PP_i + H_2O	→	2 P_i (mesophyll)
AMP + ATP	→	2ADP
Net: CO_2 (mesophyll) + ATP + 2 H_2O	→	CO_2 (bundle sheath) + 2ADP + 2 P_i
Cost of concentrating CO_2 within the bundle sheath cell = 2 ATP per CO_2		

Note: As shown in reaction 1 of Table 8.3, the H_2O and CO_2 shown in the first line of this table actually react with phosphoenolpyruvate as HCO_3^-.
P_i and PP_i stand for inorganic phosphate and pyrophosphate, respectively.

CRASSULACEAN ACID METABOLISM

This pathway worked out by Ranson and Thomas (1960) and Rouham, Vines and Black (1973) is found in succulents, mostly members of Crassulaceae (Bryophylum and Sedum) and a few members of Bromeliaceae, such as pineapple. It is a device designed to meet the pressures of heavy transpiration, arising from their xerophytic environment. These plants obtain their CO_2 requirements during the night time when they keep their stomata open and as CO_2 build-up occurs, the cell sap turns acidic. This process is called as dark acidification. In the following daytime, the stomata remain closed, minimizing transpirational losses, but with the advent of light, the CO_2 absorbed during the preceding night is utilized for photosynthetic purposes, the process , of light deacidification then occurs. Thus, there is a, time lag between absorption and reduction of CO_2. This arrangement helps to lessen transpirational stress but is responsible for the extremely slow growth of these plants. Below is given a short account of this pathway.

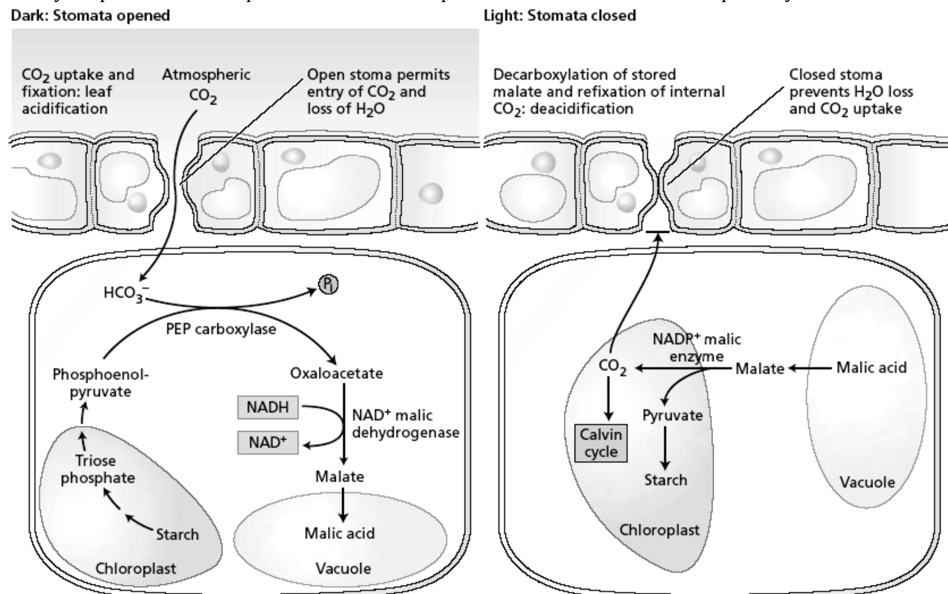
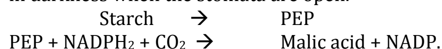


FIGURE Crassulacean acid metabolism (CAM). Temporal separation of CO_2 uptake from photosynthetic reactions: CO_2 uptake and fixation take place at night, and decarboxylation and refixation of the internally released CO_2 occur during the day. The adaptive advantage of CAM is the reduction of water loss by transpiration, achieved by the stomatal opening during the night.

Phase I : Dark acidification:

In this phase, the reserve starch is broken to phosphoenoplyruvate (PEP) through a number of respiratory reactions. PEP accept' the atmospheric C02 absorbed by the plant and produces malic acid. This happens in darkness when the stomata are open.



Starch → PEP

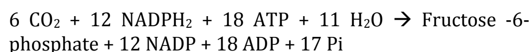
PEP + $NADPH_2$ + CO_2 → Malic acid + NADP.

Phase II: Light deacidification

During this phase, which occurs in light when the stomata are closed, malic acid is decarboxylated into pyruvic acid and CO_2. The CO_2 released is routed through Calvin cycle to synthesise a hexose. The pyruvic acid is used to build up starch whose stocks are depleted earlier.

Malic acid → Pyruvic acid + $NADPH_2$ + CO_2 in plant

Calvin cycle events:



6 CO_2 + 12 $NADPH_2$ + 18 ATP + 11 H_2O → Fructose -6-phosphate + 12 NADP + 18 ADP + 17 Pi

The assimilatory power needed for Calvin cycle is provided by the events of light deacidification.

PRINCIPLE OR LAW OF LIMITING FACTORS

Optimum value of a factor is never constant. It depends upon the magnitude of other factors. We may continue to increase the magnitude of one or more factors without influencing the rate of reaction. In such cases it is found that a factor called limiting factor is holding the balance. A limiting factor is defined as a factor which is deficient to such an extent that increase in its magnitude directly increases the rate of the process. The effect of limiting factors was studied by Blac1anarm in 1905. He formulated the principle of limiting factors which states that when a process is conditioned as to its rapidity by a number of separate factors, the rate of the process is limited by the pace of the slowest factor. In other words the rate of a physi-ological process is limited at a given time by one and only one factor which is deficient.

Factors Influencing Photosynthesis

External Factors

1. Carbon Dioxide: CO_2 concentration of the atmosphere is 0.03% or 300 ppm. It is a limiting factor as the available CO_2 concentration is lower than the optimum for photosynthesis. Increase in its concentration upto 0.1% increases the rate of photosynthesis in most land plants. A decline is observed beyond it. When CO_2 concentration is reduced, there comes a point at which illuminated plant parts stop absorbing carbon dioxide from their environment. It is known as **CO_2 compensation point** or threshold value. At this value CO_2 fixed by photosynthesis is equal to CO_2 evolved in respiration and photorespiration. *The CO_2 compensation point reflects the balance between photosynthesis and respiration as a function of CO_2 concentration, and the light compensation point reflects that balance as a function of photon flux.*

2. Light Intensity: Plants are broadly classified into two groups depending upon their inability or ability to tolerate high light intensity-shade plants (sciophytes, e.g., Oxalis) and sun plants (heliophytes)

At low light intensity the rate of photosynthesis is reduced. There is a point in light intensity where there is no gaseous exchange in photosynthesis. It is called light compensation point. Light compensation point is defined as a point in light intensity when no gaseous exchange is observed between the environment and the photosynthetic organ illuminated by that light intensity. A plant cannot survive for long at compensation point because there is a net loss of organic matter due to respiration of non-green organs and respiration in dark.

The light intensity at which a plant can achieve maximum amount of photosynthesis is called saturation point. Its value is 800-1000 ft candles (10% of full sunlight) in shade plants, 50-70% of full sunlight in C_3 sun plants and upto 200% of full sunlight in C_4 sun plants, (e.g., Sugarcane).

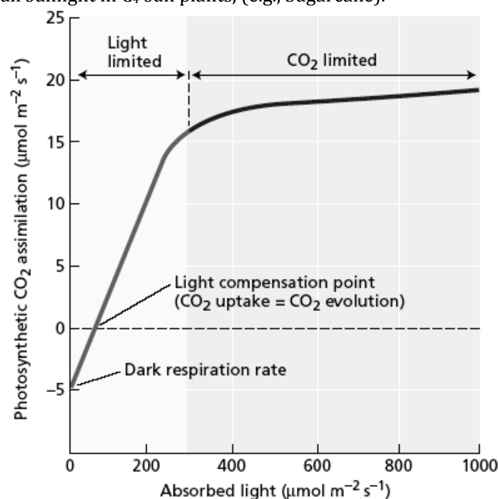


FIGURE Response of photosynthesis to light in a C_3 plant. In darkness, respiration causes a net efflux of CO_2 from the plant. The light compensation point is reached when photosynthetic CO_2 assimilation equals the amount of CO_2 evolved by respiration. Increasing light above the light compensation point proportionally increases photo-synthesis indicating that photosynthesis is limited by the rate of electron transport, which in turn is limited by the amount of available light. This portion of the curve is referred to as light-limited. Further increases in photosyn-thesis are eventually limited by the carboxylation capacity of rubisco or the metabolism of triose phosphates. This part of the curve is referred to as CO_2 limited.

Beyond saturation point (it is seldom realised in nature in C4 sun plants) the rate of photosynthesis begins to decline. The phenomenon is called solarisation. It is due to (i) Photo-inhibition due to reduction in hydration and closure of stomata (ii) Photo-oxidation or oxidation of photosynthetic pigments, intermediates and enzymes.

3. Quality of Light: Maximum 'photosynthesis occurs in blue-violet and red regions of the light spectrum where most of the absorption is carried out by chlorophylls. Red light favors carbohydrate accumulation while blue light stimulates protein: synthesis. Minimum photosynthesis occurs in the green wavelengths. Plants 'growing under the canopy of other trees receive very little red and blue-violet light because of its absorption by leaves of the canopy. They receive more of green light that is transmitted through the tree. leaves. As a result the rate of photosynthesis of herbs, shrubs and ot:4er undergrowths in a forest is comparatively low. Ultraviolet rays- are harmful.

4. Duration of Light: Continuous photosynthesis can occur in continuous illumination without any.harm to the plant (Bohning, 1949) though the rate of photosynthesis may slightly decline after six days.

5. Temperature: It does not influence light reactions of photosynthesis but affects the enzyme controlled dark reactions. The minimum temperature at which most plants start photosynthesis is 0°-5°C but it can be as low as-20°C for lichens and -35°C for some gymnosperms. The maximum temperature at which photosynthesis can occur is 55°C in some desert plants and 75°C for hot spring algae. The optimum temperature is 10°-25°C for C3 plants and 30°-45°C for C4 plants. When temperature is increased from minimum to optimum, the rate of photosynthesis doubles for every l0 °C rise in temperature. Above the optimum temperature, the rate of photosynthesis shows an initial increase for short duration but later declines. This decline with time is called time factor.

6. Oxygen: Small quantity of oxygen is essential for photosynthesis except in some anaerobic bacteria. C3 plants show optimum photosynthesis at low oxygen concentration. For example, Bjorkman et al (1968) found in beans twice the rate of photosynthesis at 2.5% oxygen as compared to normal atmospheric concentration. The possible reasons are (i) Oxygen takes part in oxidation of photosynthetic pigments, intermediates and enzymes in the presence of strong light (photo-oxidation). (ii) Oxygen is a strong quencher of excited state of chlorophyll. (iii) Oxygen competes with C02 for reducing power. However, this effect is not known in C4 plants. (iv) It converts RuBP-carboxylase to RuBP-oxygenase. At a very high oxygen content the rate of photosynthesis begins to decline in all plants. The phenomenon is called **Warburg effect**.

7. Water: Water supplies H^+ and electrons for carbon dioxide fixation. However, .less than 1°/" of the total water absorbed is utilized in photosynthesis. The rest is lost in transpiration. Even a slight increase in transpiration reduces the leaf hydration that cuts down photosynthesis by causing stomatal closure and hence decreased CO_2 absorption, loss of leaf turgidity, reduced absorption of solar radiations and decrease enzymatic activity. Thus photosynthesis is more sensitive to dehydration than any other metabolic process.

8. Air Pollutants: Dust and smoke particles present in the atmosphere reduce photosynthesis by reducing light penetration and forming a layer over the plants. Sulphur dioxide, nitrogen oxides, hydrogen fluorides and other air pollutants also decrease photosynthesis.

9. Minerals: Magnesium is a component of chlorophyll. Fe, Cu and Mn are required for synthesis of chlorophyll. Mn and Cl are linked to photolysis of water. P as phosphate is es-sential for ATP synthesis. Enzyme activators of photosynthesis include potassium and sulphur. Lower availability of any of these minerals reduces rate of photosynthesis.

Internal or Plant factors.

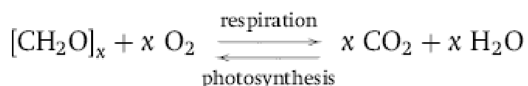
1. Age: As a leaf develops, the rate of photosynthesis rises with the age till it becomes maximum at full maturity. Afterwards the rate of photosynthesis begins to decline.

B. Respiration: Citric acid cycle; plant mitochondrial electron transport and ATP synthesis; alternate oxidase

Respiration: the oxidative breakdown of organic compounds

1 The overall process and respiratory substrates

Earlier in this chapter it was discussed how respiration counterbalances photosynthesis in the biosphere, oxidizing the C fixed in photosynthesis to CO_2 and water again. Most of the respiration is aerobic and utilizes the O_2 produced in photosynthesis, so that the O_2 is recycled as well. The primary function of respiration is to provide for the energy needs of living cells: some of the potential energy of the oxidizable substrates is conserved in ATP. Respiration is sometimes described as being 'the reverse of photosynthesis'. Chemically, the overall result of respiration is the reverse of that of photosynthesis; taking carbohydrate as the end product of photosynthesis or the substrate of respiration, one can write



$$[CH_2O]_x + x\ O_2 \underset{\text{photosynthesis}}{\overset{\text{respiration}}{\rightleftharpoons}} x\ CO_2 + x\ H_2O$$

With respect to the reaction mechanism, however, respiration as a whole is not the exact reverse of photosynthesis, although there are many common intermediates, and some reactions do run in the reverse direction in the two processes. Quantitatively, the amount of photosynthesis by a plant must exceed its respiration or a positive mass balance would be impossible. It has been estimated that a flowering plant respires daily 30 to 70% of its photosynthate, not counting any photorespiratory losses that may have occurred.

In most plant tissues, carbohydrate is the main respiratory substrate, entering the oxidative pathways as hexose sugars. Such sugars are metabolically reactive and are not stored in cells in large amounts. Carbohydrate is stored as polysaccharides of which starch, a glucose polymer, is the most common; it is insoluble and forms starch grains in plastids. Some species store fructosans, soluble polymers of fructose, in vacuoles. The disaccharide sucrose is the main vacuolar store in yet other plants, and sucrose is also the most common form in which carbohydrate is transported in plants. All these more complex carbohydrates have to be hydrolysed to their hexose monomers for respiration. Many seeds store lipids as oil and this can serve as respiratory substrate, although most of the lipid store is converted to carbohydrate before being respired. Protein is not commonly utilized as a respiratory substrate, but C skeletons from amino acids can enter the respiratory pathways. During starvation, or senescence, and during reserve mobilization in seed storage tissues, there is large-scale hydrolysis of proteins and respiration of at least part of the amino acid pool.

Much of the basic biochemistry of respiration is common to organisms from all the living kingdoms, though some details may be specific to plants. With reference to photosynthetic organisms, the respiration which passes through the universal respiratory pathways, as described below, is often termed dark respiration, to distinguish it from the unique, photosynthesis-linked photorespiration.

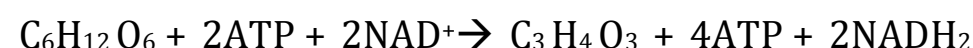
The so-called dark respiration still proceeds in photosynthetic tissues in the light. As in the case of photosynthesis, which proceeds in two stages, one can distinguish two stages in respiration: the stage of breakdown of substrate, which yields CO_2 and reduced coenzymes, NAD(P)H; and the stage of terminal oxidation, which achieves oxidation of the reduced coenzymes. Both of these stages yield ATP, but by far the greater proportion is produced during terminal oxidation.

2 Pathways of substrate breakdown

Glycolysis

The word glycolysis means 'sugar breakdown'; it brings about the oxidative breakdown of glucose to pyruvate, as illustrated in Fig. 1 . The glucose is first converted to fructose-1,6-bisphosphate by phosphorylation at the expense of ATP, catalysed by hexokinase, followed by isomerization and a second phosphorylation by phosphofructokinase. The fructose-1,6-bisphosphate is cleaved by aldolase to the two triose sugar phosphates, 3–phosphoglyceraldehyde and dihydroxyacetone-3 -phosphate. Now comes the oxidative reaction: the 3-phosphoglyceraldehyde is oxidized by glyceraldehyde-phosphate dehydrogenase to 1,3 -phosphoglycerate (PGA) and the coenzyme NAD^+ is reduced. The 1, 3 -PGA donates a phosphate group to ADP to synthesize ATP, catalysed by PGA kinase, a process known as substrate level phosphorylation.

After some molecular rearrangements, phosphoenolpyruvate, PEP, is formed and pyruvate kinase catalyses a second substrate level phosphorylation to give the end product of glycolysis, pyruvate. Since all the reactions from the oxidation step onwards proceed twice per molecule of glucose, 4 ATP per 1 glucose can be formed; but 2 ATP are used to prime the system, so that the net gain is 2 ATP per 1 glucose:



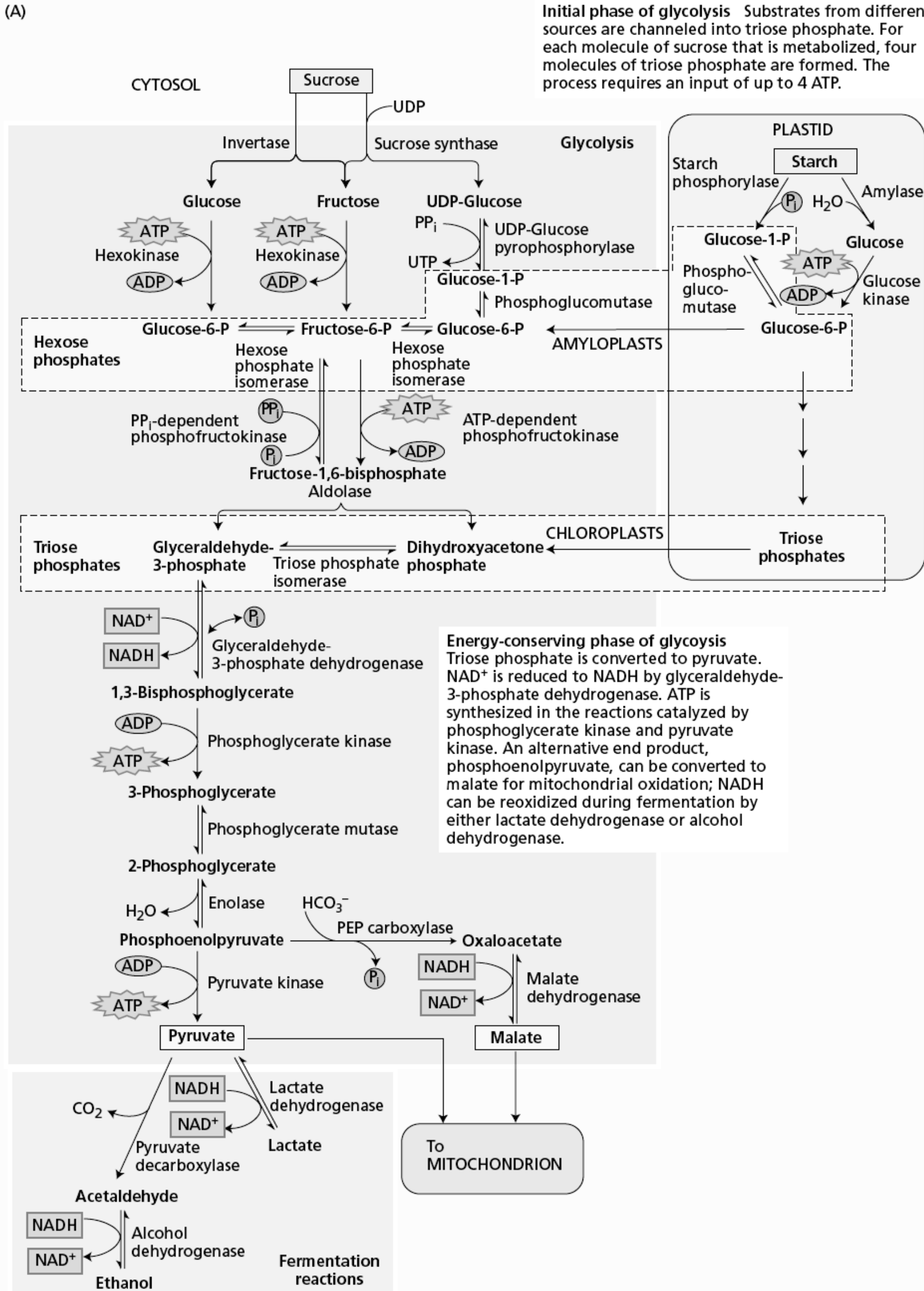
$$C_6H_{12}O_6 + 2ATP + 2NAD^+ \rightarrow C_3H_4O_3 + 4ATP + 2NADH_2$$

All the C of the glucose is still in organic combination, i.e. no CO_2 has been evolved and most of the potential energy of the glucose is still present in the pyruvate and the NADH; the net gain of 2 ATP represents a very small percentage of the potential total energy. It may also be noted that no O_2 has been used: glycolysis is an anaerobic pathway.

Substrates can enter the glycolytic pathway at several points. Fructose-6-phosphate may come from the hydrolysis of fructans or sucrose, or from the PPP. Triose sugar phosphates are transported out of chloroplasts in the light and may also be derived from the PPP. Starch hydrolysis by the starch phosphorylase enzyme produces glucose-1-phosphate, which is easily isomerized to glucose- 6-phosphate. When such phosphorylated sugars enter glycolysis, one or both of the priming reactions with ATP is/are bypassed and the ATP gain is correspondingly greater. Intermediates can also be withdrawn into other metabolic sequences from the glycolytic pathway at various points before pyruvate is produced.

The complete glycolytic sequence is located in the cytosol. In plant cells, however, isozymes of all the glycolytic enzymes are additionally found in plastids. (Isozymes are variants of an enzyme, catalysing the same reaction, but differing slightly in structure and properties such as substrate affinity; isozymes may be coded by different genes, or their differences may result from post-transcriptional or post-translational modifications.) The function of glycolysis in plastids appears to be the production of pyruvate for fatty

acid biosynthesis, a process confined to plastids in plant cells and particularly active in seed tissues synthesizing oil as nutrient store. Photosynthetic CO_2 metabolism involves several reactions catalysed by glycolytic isozymes, but in the opposite direction to respiratory reactions. For instance, in the C_3 cycle chloroplast aldolase catalyses the condensation of triose phosphates to fructose-1,6-bisphosphate; in glycolysis, cytosolic aldolase cleaves the sugar.



THE PENTOSE PHOSPHATE PATHWAY, PPP

This reaction series is also known as the hexose monophosphate shunt, or as the oxidative pentose pathway, OPP, to distinguish it from an alternative name for the C_3 cycle, which is sometimes termed the reductive pentose phosphate pathway. The PPP is located in the cytosol. It is outlined in Fig. 2.

The starting substrate for the PPP is glucose-6-phosphate (or glucose followed by the hexokinase reaction). The PPP commences with the oxidation of glucose-6-phosphate to 6-phosphogluconate, followed by an oxidative decarboxylation of the gluconate to ribulose- 5-phosphate with the release of CO_2 . The respective enzymes for these two reactions are glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase; the coenzyme which receives the H equivalents from both reactions is $NADP^+$. The ribulose-5-phosphate is recycled to glucose-6-phosphate:

6 ribulose-5-phosphate → 5 glucose-6-phosphate

The recycling reactions here are largely a reversal of the C_3 cycle reactionswhich regenerate the CO_2 acceptor ribulose-1,5-bisphosphate, but no ATP is expended and the chloroplast and the cytosol each has a distinctive set of isozymes, just as for glycolytic ones.

The balance sheet for the PPP is shown in Equations; to be able to work with whole molecules, one must start with 6 hexose (C6) molecules. Phosphates have been omitted for simplicity.

$$6\ C_6 + 12\ NADP^+ \longrightarrow 6\ CO_2 + 5\ C_6 + 12\ NADPH + 12\ H^+$$

This reduces to:

$$C_6 + 12\ NADP^+ \longrightarrow 6\ CO_2 + 12\ NADPH + 12\ H^+$$

The PPP thus does achieve the complete oxidation of glucose to CO_2 , but without ATP formation; there is no substrate-level phosphorylation involved. The major function of the PPP is thought to be the provision of NADPH for reductive biosyntheses, e.g. lipid formation, and for the production of metabolic intermediates; the pentose sugars can be utilized for nucleotide synthesis. (Some of the NADPH may be oxidized by mitochondria with ATP formation.

The oxidative pentose phosphate pathway plays several roles in plant metabolism:

• The product of the two oxidative steps is NADPH, and this NADPH is thought to drive reductive steps associated with various biosynthetic reactions that occur in the cytosol. In nongreen plastids, such as amyloplasts, and in chloroplasts functioning in the dark, the pathway may also supply NADPH for biosynthetic reactions such as lipid biosynthesis and nitrogen assimilation.

• Because plant mitochondria are able to oxidize cytosolic NADPH via an NADPH dehydrogenase localized on the external surface of the inner membrane, some of the reducing power generated by this pathway may contribute to cellular energy metabolism; that is, electrons from NADPH may end up reducing O_2 and generating ATP.

• The pathway produces ribose-5-phosphate, a precursor of the ribose and deoxyribose needed in the synthesis of RNA and DNA, respectively.

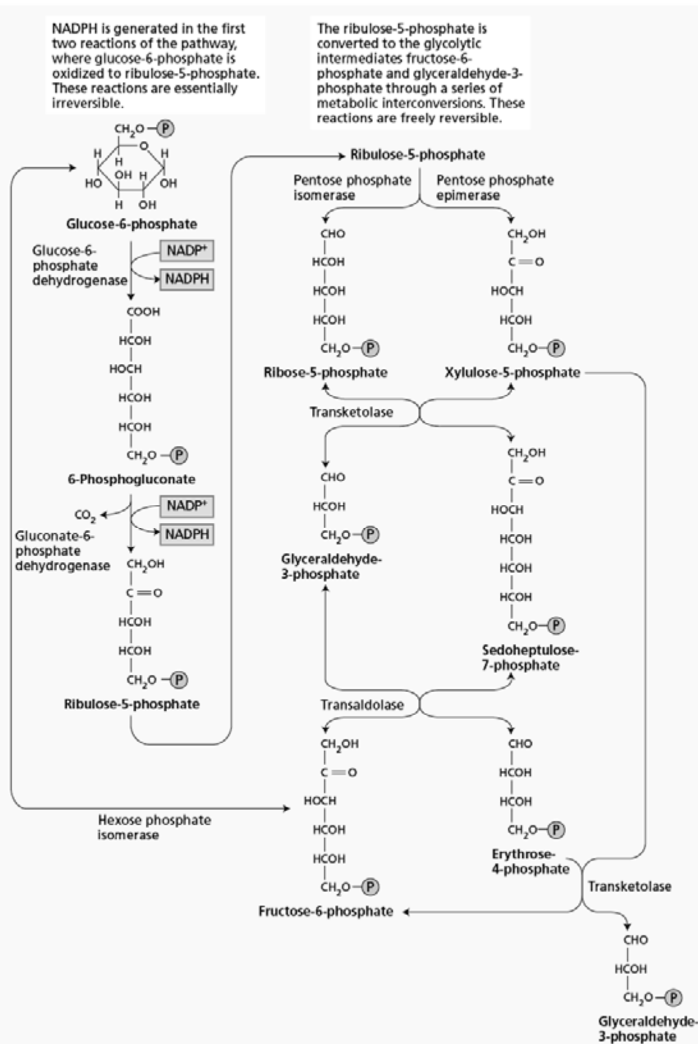
• Another intermediate in this pathway, the four-carbon erythrose-4-phosphate, combines with PEP in the initial reaction that produces plant phenolic compounds, including the aromatic amino acids and the precursors of lignin, flavonoids, and phytoalexins

• During the early stages of greening, before leaf tissues become fully photoautotrophic, the oxidative pentose phosphate pathway is thought to be involved in generating Calvin cycle intermediates.

Control of the oxidative pathway.

The oxidative pentose phosphate pathway is controlled by the initial reaction of the pathway catalyzed by glucose-6-phosphate dehydrogenase, the activity of which is markedly inhibited by a high ratio of NADPH to NADP+.

In the light, however, little operation of the oxidative pathway is likely to occur in the chloroplast because the end products of the pathway, fructose-6-phosphate and glyceraldehyde-3-phosphate, are being synthesized by the Calvin cycle. Thus, mass action will drive the nonoxidative interconversions of the pathway in the direction of pentose synthesis. Moreover, glucose-6-phosphate dehydrogenase will be inhibited during photosynthesis by the high ratio of NADPH to NADP+ in the chloroplast, as well as by a reductive inactivation involving the ferredoxin–thioredoxin System.



The Krebs cycle

The Krebs cycle is the reaction series which achieves the complete oxidation of pyruvate (coming mainly from glycolysis) to CO_2. It is located entirely and exclusively in the mitochondria. The reactions are summarized in Fig. 3

The pyruvate (with three C atoms) first loses CO_2 by oxidative decarboxylation catalysed by the enzyme pyruvate dehydrogenase, which also links the remaining 2 -C fragment, an acetyl moiety, to coenzyme A producing acetyl-CoA. This condenses with the 4 –C acid oxaloacetate to form the 6-C acid citrate. Then, as indicated in Fig. 3, there follows a series of molecular rearrangements, oxidation steps and oxidative decarboxylation steps, until the equivalent of the pyruvate has been converted to CO_2 and the oxaloacetate has been regenerated. There is one substrate-level phosphorylation producing ATP, associated with the oxidation of 2-oxoglutarate. At three steps, a molecule of water is added to the reactants. The overall final balance sheet for respiration shows water as a product, but water is also a substrate in respiration. (Compare with photosynthesis, where water is not only consumed as indicated by the overall equation, but is also a product.) The 5 pairs of H equivalents removed from the substrates in the Krebs cycle reduce NAD^+, except for the succinate oxidation step, where no coenzyme is involved. The enzymes are present in the mitochondrial matrix, but succinate dehydrogenase is again an exception, being bound to the crista membrane of the mitochondrion.

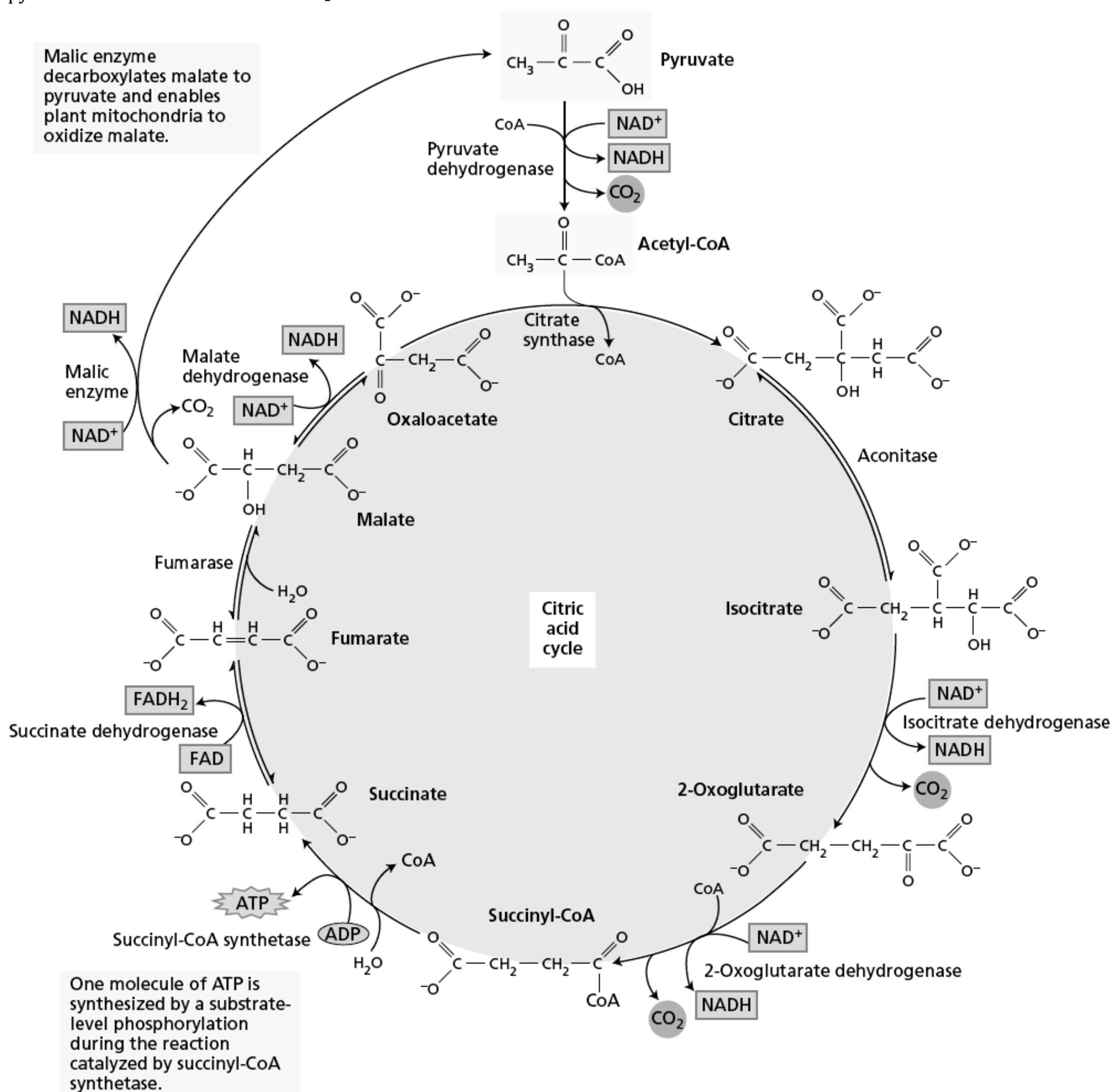


FIGURE **Reactions and enzymes of the plant citric acid cycle. Pyruvate is completely oxidized to three molecules of CO2. The electrons released during these oxidations are used to reduce four molecules of NAD+ to NADH and one molecule of FAD to FADH2.**

Between them the Krebs cycle and glycolysis can carry out the complete oxidation of glucose. With 2 [H] representing the pairs of H equivalents removed from substrates, one can write

The H equivalents can be oxidized in the terminal oxidation reactions using molecular O_2 , and producing 12 H_2O per 1 molecule glucose.

Like glycolysis and the PPP, the Krebs cycle can oxidize intermediates fed in at any place in the sequence; its role in this respect is discussed later. The Krebs cycle is also an important source for metabolic intermediates. Malate, oxaloacetate and 2- oxoglutarate are C skeletons for amino acids. Running down of the cycle by removal of intermediates is prevented by the occurrence of anaplerotic reactions which replenish it. One such reaction is the carboxylation of PEP by PEP carboxylase to give oxaloacetate; this is of course the initial reaction in C_4 and CAM photosynthesis, but it takes place also in non-photosynthetic cells, at a lower rate. Malate can be produced by malic enzyme from pyruvate, CO_2 and NADPH.

The Citric Acid Cycle of Plants Has Unique Features

The citric acid cycle reactions outlined in Figure are not all identical with those carried out by animal mitochondria.

For example, the step catalyzed by succinyl-CoA synthetase **produces ATP in plants and GTP in animals**. A feature of the plant citric acid cycle that is absent in many other organisms is the significant activity of NAD+ malic enzyme, which has been found in the matrix of all plant mitochondria analyzed to date. This enzyme catalyzes the oxidative decarboxylation of malate:

The presence of NAD^+ malic enzyme enables plant mitochondria to operate alternative pathways for the metabolism of PEP derived from glycolysis. As already described, malate can be synthesized from PEP in the cytosol via the enzymes PEP carboxylase and malate dehydrogenase. Malate is then transported into the mitochondrial matrix, where NAD+ malic enzyme can oxidize it to pyruvate. This reaction makes possible the complete net oxidation of citric acid cycle intermediates such as malate or citrate.

Alternatively, the malate produced via the PEP carboxylase can replace citric acid cycle intermediates used in biosynthesis. Reactions that can replenish intermediates in a metabolic cycle are known as *anaplerotic.* For example, export of 2-oxoglutarate for nitrogen assimilation in the chloroplast will cause a shortage of malate needed in the citrate synthase reaction. This malate can be replaced through the PEP carboxylase pathway.

The presence of an alternative pathway for the oxidation of malate is consistent with the observation that many plants, in addition to those that carry out crassulacean acid metabolism, store significant levels of malate in their central vacuole.

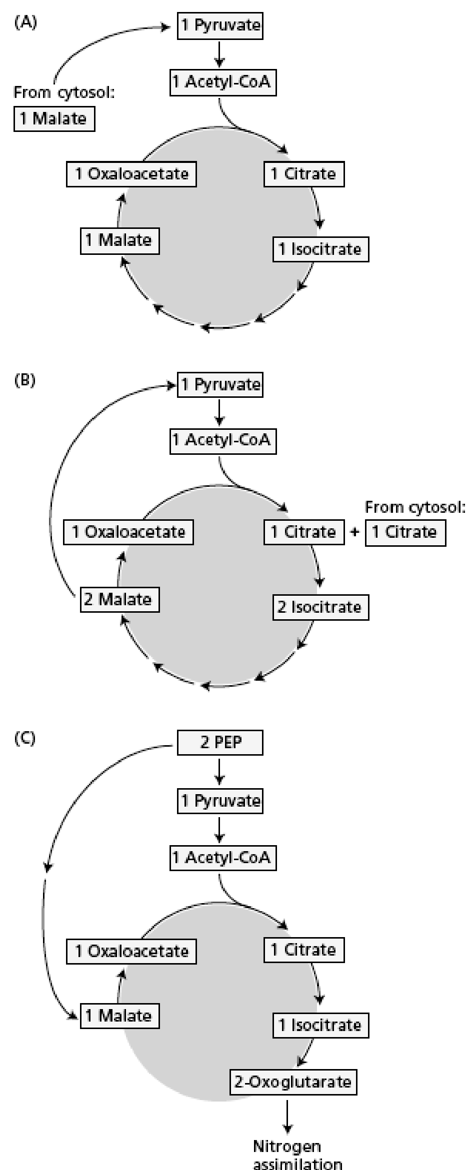


FIGURE Malic enzyme and PEP carboxylase provide plants with metabolic flexibility for the metabolism of phosphoenolpyruvate. Malic enzyme makes it possible for plant mitochondria to oxidize both malate (A) and citrate (B) to CO_2 without involving pyruvate delivered by glycolysis. The joint action of PEP carboxylase and pyruvate kinase can convert glycolytic PEP to 2-oxoglutarate, which is used for nitrogen assimilation (C).

ELECTRON TRANSPORT AND ATP SYNTHESIS AT THE INNER MITOCHONDRIAL MEMBRANE

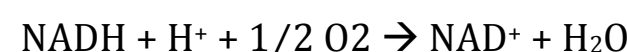
ATP is the energy carrier used by cells to drive living processes, and chemical energy conserved during the citric acid cycle in the form of NADH and FADH2 (redox equivalents with high-energy electrons) must be converted to ATP to perform useful work in the cell. This O2-dependent process, called **oxidative phosphorylation**, occurs in the inner mitochondrial membrane.

Although fundamentally similar in all aerobic cells, the electron transport chain of plants (and fungi) contains multiple NAD(P)H dehydrogenases and an alternative oxidase not found in mammalian mitochondria.

The Electron Transport Chain Catalyzes a Flow of Electrons from NADH to O_2

For each molecule of sucrose oxidized through glycolysis and the citric acid cycle pathways, 4 molecules of NADH are generated in the cytosol and 16 molecules of NADH plus 4 molecules of $FADH_2$ (associated with succinate dehydrogenase) are generated in the mitochondrial matrix.

These reduced compounds must be reoxidized or the entire respiratory process will come to a halt. The electron transport chain catalyzes an electron flow from NADH (and $FADH_2$) to oxygen, the final electron acceptor of the respiratory process. For the oxidation of NADH, the overall two-electron transfer can be written as follows:



$$NADH + H^+ + 1/2\ O2 \rightarrow NAD^+ + H_2O$$

The electron transport chain of plants contains the same set of electron carriers found in mitochondria from other organisms. The individual electron transport proteins are organized into four multiprotein complexes (identified by Roman numerals I through IV), all of which are localized in the inner mitochondrial membrane:

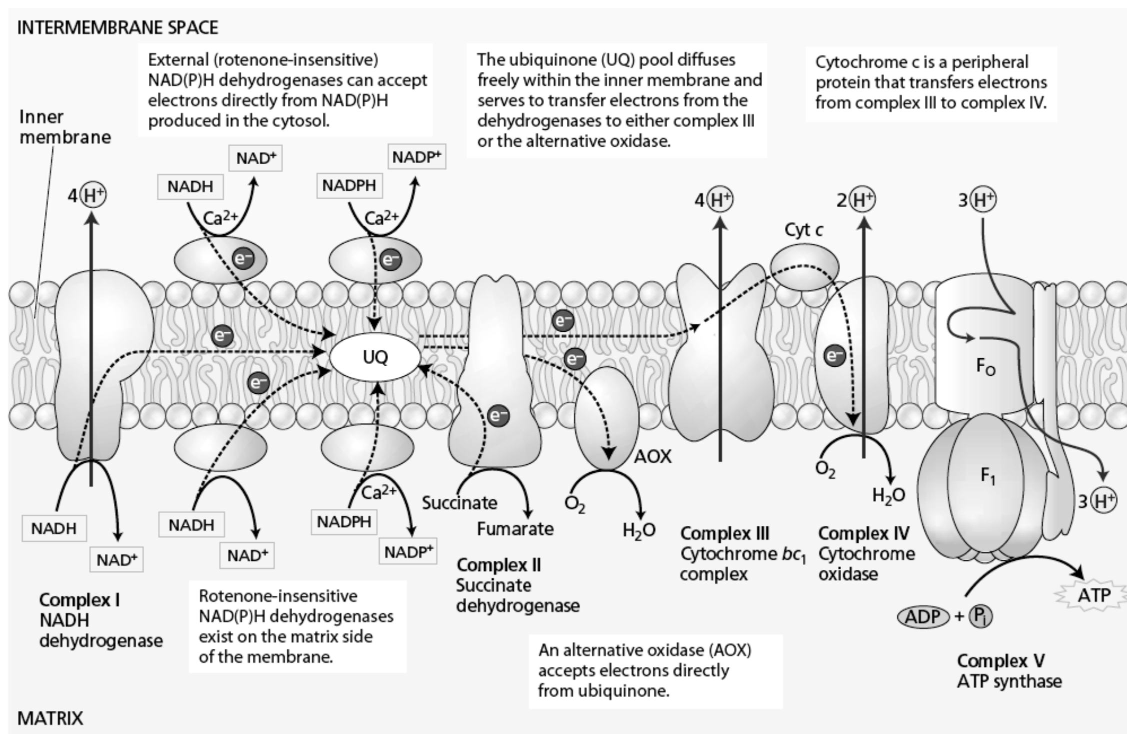


FIGURE Organization of the electron transport chain and ATP synthesis in the inner membrane of plant mitochondria. In addition to the five standard protein complexes found in nearly all other mitochondria, the electron transport chain of plant mitochondria contains five additional enzymes marked in green. None of these additional enzymes pumps protons. Specific inhibitors, rotenone for complex I, antimycin for complex III, cyanide for complex IV, and salicylhydroxamic acid (SHAM) for the alternative oxidase, are important tools to investigate the electron transport chain of plant mitochondria.

Complex I (NADH dehydrogenase). Electrons from NADH generated in the mitochondrial matrix during the citric acid cycle are oxidized by complex I (an NADH dehydrogenase). The electron carriers in complex I include a tightly bound cofactor (flavin mononucleotide [FMN], which is chemically similar to FAD) and several iron–sulfur centers. Complex I then transfers these electrons to ubiquinone. Four protons are pumped from the matrix to the intermembrane space for every electron pair passing through the complex.

Ubiquinone, a small lipid-soluble electron and proton carrier, is located within the inner membrane. It is not tightly associated with any protein, and it can diffuse within the hydrophobic core of the membrane bilayer.

Complex II (succinate dehydrogenase). Oxidation of succinate in the citric acid cycle is catalyzed by this complex, and the reducing equivalents are transferred via the $FADH_2$ and a group of iron–sulfur proteins into the ubiquinone pool. This complex does not pump protons.

Complex III (cytochrome bc1 complex). This complex oxidizes reduced ubiquinone (ubiquinol) and transfers the electrons via an iron–sulfur center, two *b*-type cytochromes (*b*565 and *b*560), and a membrane-bound cytochrome *c*1 to cytochrome *c*. Four protons per electron pair are pumped by complex III.

Cytochrome *c* is a small protein loosely attached to the outer surface of the inner membrane and serves as a mobile carrier to transfer electrons between complexes III and IV.

Complex IV (cytochrome c oxidase). This complex contains two copper centers (CuA and CuB) and cytochromes *a* and *a*3. Complex IV is the terminal oxidase and brings about the four-electron reduction of O_2 to two molecules of H_2O.

Two protons are pumped per electon pair. Both structurally and functionally, ubiquinone and the cytochrome *bc*1 complex are very similar to plastoquinone and the cytochrome *b*6 *f* complex, respectively, in the photosynthetic electron transport chain.

Some Electron Transport Enzymes Are Unique to Plant Mitochondria

In addition to the set of electron carriers described in the previous section, plant mitochondria contain some components not found in mammalian mitochondria. Note that none of these additional enzymes pump protons and that energy conservation is therefore lower whenever they are used:

• Two NAD(P)H dehydrogenases, both Ca_2^+-dependent, attached to the outer surface of the inner membrane facing the intermembrane space can oxidize cytosolic NADH and NADPH. Electrons from these external NAD(P)H dehydrogenases—NDex(NADH) and NDex(NADPH)—enter the main electron transport chain at the level of the ubiquinone pool.

• Plant mitochondria have two pathways for oxidizing matrix NADH. Electron flow through complex I, described in the previous section, is sensitive to inhibition by several compounds, including rotenone and piericidin. In addition, plant mitochondria have a rotenone-resistant dehydrogenase, NDin(NADH), for the oxidation of NADH derived from citric acid cycle substrates. The role of this pathway may well be as a bypass being engaged when complex I is overloaded, such as under photorespiratory conditions.

• An NADPH dehydrogenase, NDin(NADPH), is present on the matrix surface. Very little is known about this enzyme.

• Most, if not all, plants have an "alternative" respiratory pathway for the reduction of oxygen. This pathway involves the so-called alternative oxidase that, unlike cytochrome *c* oxidase, is insensitive to inhibition by cyanide, azide, or carbon monoxide.

ATP Synthesis in the Mitochondrion Is Coupled to Electron Transport

In oxidative phosphorylation, the transfer of electrons to oxygen via complexes I to IV is coupled to the synthesis of ATP from ADP and Pi via the ATP synthase (complex V). The number of ATPs synthesized depends on the nature of the electron donor. In experiments conducted with the use of isolated mitochondria, electrons derived from internal (matrix) NADH give ADP:O ratios (the number of ATPs synthesized per two electrons transferred to oxygen) of 2.4 to 2.7. Succinate and externally added NADH each give values in the range of 1.6 to 1.8, while ascorbate, which serves as an artificial electron donor to cytochrome *c*, gives values of 0.8 to 0.9. Results such as these (for both plant and animal mitochondria) have led to the general concept that there are three sites of energy conservation along the electron transport chain, at complexes I, III, and IV.

Aerobic Respiration Yields about 60 Molecules of ATP per Molecule of Sucrose

The complete oxidation of a sucrose molecule leads to the net formation of

• 8 molecules of ATP by substrate-level phosphorylation (4 during glycolysis and 4 in the citric acid cycle)
• 4 molecules of NADH in the cytosol
• 16 molecules of NADH plus 4 molecules of FADH2 (via succinate dehydrogenase) in the mitochondrial matrix

On the basis of theoretical ADP:O values, a total of approximately 52 molecules of ATP will be generated per sucrose by oxidative phosphorylation. The result is a total of about 60 ATPs synthesized per sucrose (Table).

The maximum yield of cytosolic ATP from the complete oxidation of sucrose to CO_2 via aerobic glycolysis and the citric acid cycle

Part reaction		ATP per sucrose[a]
Glycolysis		
4 substrate-level phosphorylations		4
4 NADH	4 × 1.5	6
Citric acid cycle		
4 substrate level phosphorylations		4
4 $FADH_2$	4 × 1.5	6
16 NADH	16 × 2.5	40
Total		60

Using 50 kJ mol^{-1} (12 kcal mol^{-1}) as the actual free energy of formation of ATP in vivo, we find that about 3010 kJ mol^{-1} (720 kcal mol^{-1}) of free energy is conserved in the form of ATP per mole of sucrose oxidized during aerobic respiration. This amount represents about 52% of the standard free energy available from the complete oxidation of sucrose; the rest is lost as heat. This is a vast improvement over the conversion of only 4% of the energy available in sucrose to ATP that is associated with fermentative metabolism.

Plants Have Several Mechanisms That Lower the ATP Yield

As we have seen, a complex machinery is required for a high efficiency of energy conservation in oxidative phosphorylation. So it is perhaps surprising that plant mitochondria have several functional proteins that reduce this efficiency. Probably plants are less limited by the energy supply (sunlight) than by other factors in the environment (e.g., access to nitrogen or phosphate). As a consequence, adaptational flexibility may be more important than energetic efficiency.

In the following subsections we will discuss the role of the nonphosphorylating mechanisms and their possible usefulness in the life of the plant.

The alternative oxidase. If cyanide (1 m*M*) is added to actively respiring animal tissues, cytochrome *c* oxidase is inhibited and the respiration rate quickly drops to less than 1% of its initial level. However, most plant tissues display a level of cyanide-resistant respiration that can represent 10 to 25%, and in some tissues up to 100%, of the uninhibited control rate. The enzyme responsible for this oxygen uptake has been identified as a cyanide-resistant oxidase component of the plant mitochondrial electron transport chain called the **alternative oxidase.**

Electrons feed off the main electron transport chain into the alternative pathway at the level of the ubiquinone pool. The alternative oxidase, the only component of the alternative pathway, catalyzes a four-electron reduction of oxygen to water and is specifically inhibited by several compounds, most notably **salicylhydroxamic acid (SHAM).**

When electrons pass to the alternative pathway from the ubiquinone pool, two sites of proton pumping (at complexes III and IV) are bypassed. Because there is no energy

conservation site in the alternative pathway between ubiquinone and oxygen, the free energy that would normally be conserved as ATP is lost as heat when electrons are shunted through the alternative pathway.

How can a process as seemingly energetically wasteful as the alternative pathway contribute to plant metabolism?

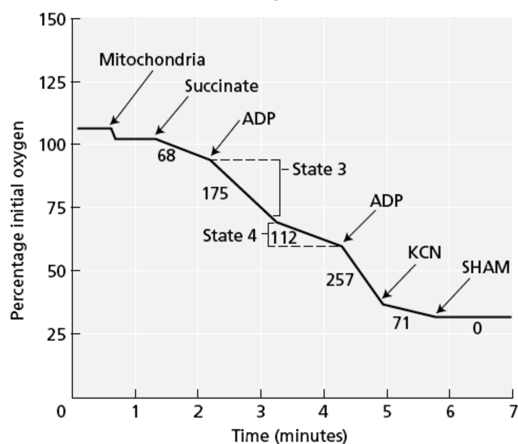
One example of the functional usefulness of the alternative oxidase is its activity during floral development in certain members of the Araceae (the arum family)—for example, the voodoo lily (*Sauromatum guttatum*). Just before pollination, tissues of the clublike inflorescence, called the *appendix*, which bears male and female flowers, exhibit a dramatic increase in the rate of respiration via the alternative pathway. As a result, the temperature of the upper appendix increases by as much as 25°C over the ambient temperature for a period of about 7 hours.

During this extraordinary burst of heat production, certain amines, indoles, and terpenes are volatilized, and the plant therefore gives off a putrid odor that attracts insect pollinators. **Salicylic acid, a phenolic compound related to aspirin,** has been identified as the chemical signal responsible for initiating this thermogenic event in the voodoo lily.

In most plants, however, both the respiratory rates and the rate of cyanide-resistant respiration are too low to generate sufficient heat to raise the temperature significantly, so what other role(s) does the alternative pathway play?

1. Addition of succinate initiates mitochondrial electron transfer, which is measured with an oxygen electrode as the rate of oxygen reduction (to H_2O).

2. Addition of cyanide inhibits electron flow through the main cytochrome pathway and only allows electron flow to oxygen through the alternative, cyanide-resistant pathway, which is subsequently inhibited by the addition of SHAM.



3. Addition of ADP stimulates electron transfer (state 3) by facilitating dissipation of the electrochemical proton gradient. The rate is higher after the second ADP addition because of activation of succinate dehydrogenase.

4. When all the ADP has been converted to ATP, electron transfer reverts to a lower rate (state 4).

FIGURE Regulation of respiratory rate by ADP during succinate oxidation in isolated mitochondria from mung bean (*Vigna radiata*). The numbers below the traces are the rates of oxygen uptake expressed as O_2 consumed (nmol min^{-1} mg $protein^{-1}$).

It has been suggested that the alternative pathway can function as an "energy overflow" pathway, oxidizing respiratory substrates that accumulate in excess of those needed for growth, storage, or ATP synthesis. This view suggests that electrons flow through the alternative pathway only when the activity of the main pathway is saturated. Such saturation is reached in the test tube in state 4; in vivo, saturation may occur if the respiration rate exceeds the cell's demand for ATP (i.e., if ADP levels are very low). However, it is now clear that the alternative oxidase can be active before the cytochrome pathway is saturated. Thus the alternative oxidase makes it possible for the mitochondrion to adjust the relative rates of ATP production and synthesis of carbon skeletons for use in biosynthetic reactions.

Another possible function of the alternative pathway is in the response of plants to a variety of stresses (phosphate deficiency, chilling, drought, osmotic stress, and so on), many of which can inhibit mitochondrial respiration. By draining off electrons from the electron transport chain, the alternative pathway prevents a potential overreduction of the ubiquinone pool, which, if left unchecked, can lead to the generation of destructive reactive oxygen species such as superoxide anions and hydroxyl radicals. In this way the alternative pathway may lessen the detrimental effects of stress on respiration.

The uncoupling protein. A protein found in the inner membrane of mammalian mitochondria, the **uncoupling protein**, can dramatically increase the proton permeability of the membrane and thus act as an uncoupler. As a result, less ATP and more heat is generated. Heat production appears to be one of the uncoupling protein's main functions in mammalian cells.

It has long been thought that the alternative oxidase in plants and the uncoupling protein in mammals were simply two different means of achieving the same end. It was therefore surprising when a protein similar to the ncoupling protein was discovered in plant mitochondria. This protein is stress induced and, like the alternative oxidase, may function to prevent overreduction of the electron transport chain. It remains unclear, however, why plant mitochondria require both mechanisms.

The internal, rotenone-insensitive NADH dehydrogenase, NDin(NADH).

This is one of the multiple NAD(P)H dehydrogenases found in plant mitochondria. It has been suggested to work as a nonproton-pumping bypass when complex I is overloaded. Complex I has a higher affinity for NADH (ten times lower *K*m), than NDin(NADH). At lower NADH levels in the matrix, typically when ADP is available (state 3), complex I will dominate, whereas when ADP is rate limiting (state 4), NADH levels will increase and NDin(NADH) will be more active. The physiological importance of this enzyme is, however, still unclear.

Mitochondrial Respiration Is Controlled by Key Metabolites

The substrates of ATP synthesis—ADP and Pi—appear to be key regulators of the rates of glycolysis in the cytosol, as well as the citric acid cycle and oxidative phosphorylation in the mitochondria. Control points exist at all three stages of respiration; here we will give just a brief overview of some major features.

The best-characterized site of regulation of the citric acid cycle is at the pyruvate dehydrogenase complex, which is reversibly phosphorylated by a regulatory kinase and a phosphatase.

Pyruvate dehydrogenase is inactive in the phosphorylated state, and the regulatory kinase is inhibited by pyruvate, allowing the enzyme to be active when substrate is available. In addition, several citric acid cycle enzymes, including pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, are directly inhibited by NADH.

Lipid oxidation

Lipids are frequently stored in seeds as oil bodies (oleosomes, sphaerosomes), where they may account for over 50% of the tissue mass. The storage oils are triglycerides consisting of a glycerol molecule esterified with three long-chain fatty acids, mostly with 16 or 18 C atoms per chain. The first step in lipid oxidation is hydrolysis by lipase enzymes within the oil body to glycerol and fatty acids. Glycerol, a 3 -C sugar alcohol, is closely related to triose sugars and is converted to dihydroxyacetone-3 - phosphate, which can then enter the glycolytic pathway. The fatty acids are activated by linkage to coenzyme A and are metabolized in the glyoxysomes (specialized microbodies of oily seeds) by two metabolic sequences. Firstly, the process of β-oxidation results in the cleavage of the fatty acid chain into 2 -C fragments, acetyl-CoA. Secondly, the glyoxysomes process the acetyl-CoA via the glyoxylate cycle to succinate and this can, through a series of reactions in the cytosol and the mitochondria, finally be synthesized to sucrose. Most of the lipid stored in seeds is converted to sucrose and transported to the growing parts of the seedling. Some of the acetyl-CoA is utilized in the mitochondria of the storage cells as respiratory substrate in the Krebs cycle.

3 Interactions of pathways

In the cell, none of the pathways of respiratory substrate oxidation functions in isolation. A summary overview of all the pathways, and their interrelationships, is presented in Fig. 2 .15. The first stage, 'hydrolysis' in Fig. 4, does not involve oxidations and is not specific to respiration: the monomers produced from the polymers serve as substrates not only for respiration but for numerous other metabolic sequences. The free energy change associated with the hydrolytic reactions is minimal, less than 1% of the total available, and cannot support ATP synthesis. The 'incomplete oxidation' stage produces a small number of fairly simple organic acids and the free energy content of the substrate falls by about 33 %; some of the energy is conserved in ATP during substrate level phosphorylations, and also in NADH. The organic acids feed into the 'complete oxidation' stage, the Krebs cycle, which in association with the mitochondrial electron transport chain forms the final stage for most aerobic respiratory activity and where most of the ATP synthesis takes place. The PPP does not appear to fit the overall pattern in that it is a direct oxidative pathway; sugars metabolized exclusively by the PPP do not pass through the Krebs cycle. However, the PPP and glycolysis share common intermediates, fructose-6-phosphate and triose phosphates. Since both reaction series take place in the cytosol, it is almost inevitable that some exchange of metabolites between the two pathways should occur. If, say, there is a high demand for NADPH, the flow of material through the PPP might be boosted by intermediates from the glycolytic sequence. A large demand for pyruvate, on the other hand, could result in the channelling of PPP intermediates into glycolysis. The PPP accordingly should be considered an integral part of a glycolysis – Krebs cycle – PPP network. This network in turn interacts with the rest of cellular metabolism via interchange of metabolites, at many steps. The provision of intermediates and reduced coenzymes is just as much a function of respiration as provision of ATP; respiration, defined as a substrate breakdown process, is also the starting point of many biosyntheses.

The ATP balance sheet and energy-conversion efficiency of respiration

The theoretical balance sheet for respiratory ATP production is easily drawn up. If glucose is completely oxidized via glycolysis and the Krebs cycle, with terminal oxidation through cytochrome oxidase, one can add up the ATP molecules per 1 molecule glucose (Table 2). The NADH from glycolysis, reacting with mitochondria from the outside, is oxidized with the production of only 2 ATP per NADH, whilst mitochondrially produced coenzyme oxidation yields 3 ATP per 1 NADH. The free energy of complete oxidation of glucose is 2880 kJ mol^{-1} . The free energy of hydrolysis of ATP is highly dependent on factors such as ATP concentration and pH, but under cellular conditions is at least 42 kJ mol^{-1} . Assuming this value, a gain of 36 ATP per molecule of glucose is equivalent to an energy conservation of [3 6 x 4 2], 1512 kJ mol^{-1} , or 52 % of the total available, a very high degree of energy conservation. (The value would be pushed even higher if phosphorylated sugars enter the glycolytic pathway.

Table 2. The balance sheet for respiratory production of molecules of ATP per 1 molecule of glucose, assuming complete coupling and complete oxidation via glycolysis, Krebs cycle and cytochrome oxidase system.

Substrate level oxidation in glycolysis	4
Substrate level oxidation in Krebs cycle	2
Terminal oxidation, 2 NADH from glycolysis	4
Terminal oxidation, 8 NADH from Krebs cycle	24
Terminal oxidation, 2 succinate	4
Total produced	38
Used up in glycolysis, priming reactions	2
Net gain	36

The calculation in Table 2 is, however, based on an assumption of complete coupling of substrate breakdown and terminal oxidation to ATP synthesis. This is unlikely to be the situation in a cell, at least not in all circumstances. Any NADH which is used in reductive reactions does not yield ATP, and terminal oxidation by the alternative oxidase produces only 1 ATP per molecule of NADH oxidized. The 12 NADPH formed per molecule of glucose in the PPP could theoretically be oxidized by the mitochondria with the production of 24 ATP, but, as stated, the PPP probably provides NADPH mainly for reductive reactions rather than for terminal oxidation. It is therefore not possible to say exactly how much ATP is produced per glucose molecule in a particular situation.

Anaerobic respiration

Occurrence and endurance of anaerobiosis in plants

Flowering plants are obligate aerobes: no flowering plant can complete its life cycle without O_2 . Most flowering plant organs, except dormant seeds, are killed by anoxia (complete lack of O_2) within hours or a few days at most. Nevertheless there are also many situations in which plant

tissues regularly survive at least hypoxia, O_2 concentrations too low to support a normal level of aerobic respiration. Anaerobic respiration (fermentation) is therefore not unusual in plants. In hypoxia, both aerobic and anaerobic pathways operate simultaneously.

Germinating seeds often undergo a period of O_2 shortage during imbibition, because the testa can be highly impermeable towards O_2 ; also before the cells are fully hydrated and expanded there are hardly any air spaces between them. Respiration during this period has a high RQ, respiratory quotient, i.e. the ratio CO_2 evolution : O_2 uptake.

For aerobic respiration this ratio is unity; in anaerobic respiration, CO_2 is evolved without any O_2 uptake, raising the RQ. Analysis shows an accumulation of end products of fermentation, such as ethanol, in the seed tissues. When the emerging radicle splits the testa, the RQ falls. Complete anoxia is, however, tolerated by seeds of very few species.

Meristematic tissues carry out partly anaerobic respiration. Cells in meristematic regions are closely packed, without air spaces, making diffusion of O_2 a problem. The vascular cambium lies quite deeply within plant organs and outside it lies the phloem, a living tissue without intercellular air spaces and with a high demand for O_2 . In apical meristems the mitochondria are immature, with few cristae per unit volume, so that their capacity for terminal oxidation is low.

All these factors favour anaerobic respiration. Inside bulky organs – large fruits, tubers, thick stems and thick roots – the level of O_2 may be under 2 .5% and partially anaerobic respiration would be expected; ethanol accumulation has been detected. However, some bulky organs are well supplied with air spaces and then the problem of O_2 diffusion is much reduced.

In seeds, large organs and meristems, hypoxia is the result of the structure of the plant itself. In aquatic plants hypoxia is imposed by the environment. The solubility of O_2 in water is low. Water at 10 ^{0}C in equilibrium with normal air, which is 21% O_2 by volume, contains only about 0.80% O_2 and the solubility falls with rising temperature. Roots and rhizomes growing in mud at the bottom of a body of water, or in boggy ground, will be in an essentially anoxic environment. By daytime, green parts of submerged aquatics can respire aerobically on the O_2 evolved in photosynthesis and O_2 is then also conducted to the roots through the extensive air spaces of these plants. By night the O_2 levels in and around the plants will fall very low, especially for the parts in the muddy substratum. These parts are highly tolerant of anoxia. The marsh plant Acorus calamus grows from a rhizome and normally overwinters in a submerged resting state, with a low metabolic rate.

Explants from the resting plants, consisting of segments of rhizome with attached roots and small leaves, have survived laboratory incubation under total anoxia, in the dark, for two months. Seeds of a limited number of aquatic species can germinate in a totally anaerobic environment. Examples are rice, a few species of the grass Echinochloa found as a weed in rice fields, and the yellow water lily (*Nuphar luteum*). This is obviously an adaptation to their natural environment, where these seeds germinate at the bottom of the water. Rice and Echinochloa seedlings can survive for at least four days anaerobically.

As the above examples demonstrate, varying degrees of anaerobiosis are normal for, and tolerated by, numerous plant species or tissues. On the other hand, many species are intermittently damaged by anaerobiosis owing to flooding. Once the air spaces in the soil are filled with water, the limited amount of O_2 that is dissolved is rapidly used up by the respiration of plant roots and soil microorganisms and the roots then suffer an O_2 shortage; this can have serious consequences for an agricultural crop. An understanding of anaerobic respiration is therefore of considerable practical interest.Knowledge of how e.g. aquatic plants endure anaerobiosis might help the development of flooding-tolerant crop plants.

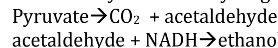
Respiratory metabolism under anaerobiosis

The respiratory pathwaywhich is functional in anaerobic plant tissues is glycolysis, just as it is in anaerobic microorganisms; this pathway is essentially an anaerobic reaction sequence. On transfer to anoxic or hypoxic conditions, the transcription and translation of most plant genes is inhibited, but there is enhanced transcription and translation of some 20 genes, a major part of which code for glycolytic enzymes: aldolase, glucose-phosphate isomerase, enolase and glyceraldehydes phosphate dehydrogenase, including some isozymes not synthesized aerobically. The pyruvate produced in glycolysis in the absence of O_2 becomes the oxidant which receives the H equivalents from glycolytic NADH and recycles it to NAD^+, which is vital for the continuance of glycolysis; cells contain only very small amounts of the coenzyme. Three pathways of fermentation have been demonstrated in plant tissues:

(1) Lactic fermentation: the enzyme lactate dehydrogenase catalyses the reaction:

pyruvate + NADH → lactate + NAD

(2) Alcoholic fermentation: firstly pyruvate decarboxylase decarboxylates the pyruvate to acetaldehyde, then this is reduced with NADH by alcohol dehydrogenase (ADH):


Pyruvate→CO_2 + acetaldehyde
acetaldehyde + NADH→ethanol

(3) Malic fermentation: malic enzyme carboxylates the pyruvate to malate:

pyruvate + CO2 + NADH → malate + NAD^+

Increased transcription of mRNA for lactate dehydrogenase, pyruvate decarboxylase and ADH occurs as a response to anaerobiosis. Why should anaerobiosis be so harmful to plants? There is still no clear answer to this, nor to what confers tolerance on such plants as show it. One serious drawback of anaerobic metabolism is the extremely low yield of ATP per unit of substrate respired. A switch from complete aerobic oxidation to complete anaerobiosis could mean a fall from 3 6 to 2 ATP produced per molecule of glucose oxidized, a decrease to 5.5%, insufficient to keep a cell alive. Even if aerobic respiration were producing only half the theoretical maximal ATP, the decrease induced by anaerobiosis would still be drastic. The low ATP yield can be offset to some degree by the increases in the rate of glycolysis which typically occur under anaerobiosis (the Pasteur effect). But increases in the rate of glycolysis induced in plant tissues by anaerobiosis are only 1.5- to 6-fold. With high rates of glycolysis there is a danger of substrate exhaustion and starvation, and also much accumulation of potentially harmful products of fermentation. Lactate and malate are acids and acidification of the cytoplasm has been cited as the main cause of cell death in some instances. Acetaldehyde, the intermediate in alcoholic fermentation, is highly toxic; ethanol is, however, tolerated comparatively well. Maize mutants deficient in an ADH gene succumb rapidly to anaerobiosis, presumably because they cannot remove the acetaldehyde. There is also, strange as it may seem, evidence of oxidative stress, with formation of hydrogen peroxide, a dangerous oxidant, from

trace amounts of O_2 . Tissue damage and death could result fromsubstrate exhaustion, acidification, lipid peroxidation and toxin accumulation, singly or in combination.

In plant tissues tolerant to anoxia or hypoxia, no basic differences in anaerobic metabolism have been detected, in comparison with sensitive material. Differences seem to be quantitative rather than qualitative; alcoholic fermentation predominates. In submerged plants, at least a sizeable proportion of the ethanol leaches out into the surrounding water, the permeability of cellular membranes towards ethanol being high. This helps to keep down the concentration of alcohol in the tissues. The Acorus calamus rhizome which overwinters in mud, lays down an abundant store of starch during the summer. Rice grains, which are very tolerant to anaerobiosis, are able to synthesize a-amylase in anoxic conditions and hence can mobilize their starch reserves. The sensitive wheat grain is unable to synthesize the enzyme and cannot mobilize starch in the absence of O_2 , but can be induced to germinate anaerobically by feeding with sucrose or glucose. These examples show that an ability to maintain an adequate supply of respiratory substrate can be a factor in survival under anaerobiosis.

Tolerance towards anaerobiosis may need induction. Rice seedlings (as distinct from the ungerminated grains), which are killed within 24 hours when suddenly exposed to complete anoxia, can survive anoxia for several days when first pretreated at low O_2 levels. During the pretreatment period, there are increases in the activities of ADH and pyruvate decarboxylase, giving the plants a greater potential for alcoholicfermentation. In maize, development of flooding tolerance involves increases in activity of several genes coding for ADH and other enzymes of anaerobic respiration.

Correlation of respiration rate with physiological activity

Respiration proceeds unceasingly in all active (i.e. non-dormant) living cells. Even when a cell is not performing any net metabolic work and is simply subsisting unchanged – say a mature pith parenchyma cell – it still requires repair and resynthesis of protoplasmic components, which are labile and in a constant state of turnover. Membrane potentials are sustained only by continued pumping of ions across membranes, requiring ATP. This aspect of cellular activity has led to the concept of maintenance respiration, required for such processes. The proportion of respiration that supports net cellular work is then termed growth respiration (synthetic respiration). More precise definitions of the terms have been attempted, while some plant physiologists have disputed the validity of any division of respiration into these components. The general concept of maintenance respiration is, however, useful as a reminder that cells must spend energy for their survival, without cessation. It is not implied that there is a biochemical distinction between growth respiration and maintenance respiration. Measurement of the proportion of maintenance respiration is even more controversial than its definition, but for plant cells, its magnitude has been estimated at up to 50% of the total.

Respiration rates are positively correlated with physiological activity. On a unit mass basis (fresh or dry mass), high rates are found in young, actively growing regions, such as growing apices, or in tissues performing metabolic work at a high rate, such as glands. To some extent this is the result of the higher ratio of living protoplasm per unit mass in such tissues; mature and metabolically more inert tissues have larger proportions of cell wall and/or vacuoles and storage materials per unit mass, and these tissue compartments do not contribute to respiratory activity. On a unit nitrogen basis, which reflects more truly the 'living' mass, differences between tissues of varying maturity and metabolic activity become less marked. But in the same tissue, respiration rate can be shown to increase with increasing activity. For instance, when roots are washed in distilled water and then transferred to a nutrient solution from which they proceed to take up ions, their respiration rate increases concomitantly with ion absorption. In nectaries, the period of rapid sugar secretion coincides with a period of rapid respiration.

The relationship between respiration and growth has been the subject of much study and speculation. As already noted, growing tissues are characterized by high rates of respiration. The quantitative relationship between growth rate and respiration rate is, however, not a simple one. On the whole, plants with higher growth rates tend to have the higher respiration rates, although there are also reports to the contrary, e.g. of faster-growing genotypes of a grass (*Lolium perenne*) having lower respiration rates. When plant systems are compared, the differences in their rates of respiration are typically less than the differences in their growth rates. In a study with nine species of grasses, for a 2 –3 -fold higher relative growth rate, RGR (for definition of RGR), the increase in respiration was only 1.4 –1.7-fold. Explanations must be sought in differences in metabolism, resulting in a greater efficiency of respiratory energy production (or utilization) in the faster-growing species. One such difference might be in the degree to which the alternative oxidase participates in the respiration of different tissues. In a study of root respiration and growth in four inbred lines of the greater plantain (Plantago major) it was found that higher RGR were associated with a higher proportion of respiration passing through the more energy-efficient cytochrome pathway as opposed to the alternative oxidase.

An understanding of relationships between growth and respiration is of great relevance to considerations of plant productivity. Respiration results in a loss of biomass, even while it is ecessary to support growth. There have been arguments as to whether it is more favourable to breed a crop with a low respiration rate, for minimal loss of biomass, or with a high rate, associated with a high RGR. It appears nowthat it is not just overall respiration rate that is relevant to productivity. To maximize productivity, one would need to maximize the efficiency with which the potential energy of the respiratory substrates is converted to usable form – ATP and reduced coenzymes. We are not yet certain wherein this efficiency lies.

Metabolic control of rates: feedback mechanisms

Respiration rate is geared to the requirements of tissues by numerous mechanisms. One of these is the cellular concentration of ADP, and the ATP : ADP ratio. Respiration produces ATP, growth and maintenance processes consume it and produce ADP, the total cellular amount of [ATP + ADP] remaining constant for prolonged periods. ADP is an essential reactant in respiration. It is directly used in substrate-level phosphorylations, and mitochondrial electron transport through cytochrome oxidase is normally coupled to oxidative phosphorylation, which requires ADP. When there is an increase in an ATP-utilizing cellular activity, more ATP per unit time is converted to ADP. The higher ADP concentration stimulates a faster rate of substrate breakdown and mitochondrial electron transport

– with a higher rate of ATP synthesis. The result is a higher rate of respiration and a higher turnover of ATP and ADP. The ratio ATP : ADP seems to be fairly constant in cells.

The effects of ATP and ADP are not only simple concentration effects. The glycolytic enzyme phosphofructokinase is inhibited by ATP, which is one of its substrates; pyruvate kinase is also inhibited by ATP. On the other hand, ADP activates pyruvate kinase. These effects would result in slowing glycolysis by an increase in ATP concentration and a speeding up by a rise in the concentration of ADP.

Accumulation of intermediates of the respiratory pathways causes inhibition of enzymes acting earlier in the pathways – the well-known phenomenon of feedback inhibition. Phosphofructokinase, one ofthe first enzymes of the glycolytic pathway, is inhibited by phosphoglycerate and by phosphoenolpyruvate. Citrate (from the Krebs cycle) inhibits both phosphofructokinase and pyruvate kinase. A fall in the demand for ATP would slow down the mitochondrial electron transport; citrate metabolism would slow down correspondingly, being dependent on the mitochondrial terminal oxidation. The accumulating citrate would cause slowing down of glycolysis. Similarly, a fall in the demand for biosynthetic intermediates would result in a build-up of these intermediates, and in an inhibition of preceding steps. Any change in requirement for ATP or/and metabolic intermediates would result in an adjustment in respiration rate, until a new equilibrium between supply and demand was established. For the PPP, the ratio of $NADP^+$: NADPH is a regulatory factor.

When metabolic pathways were first elucidated, rate control was postulated to be concentrated at a few key steps catalysed by ‘pacemaker’ enzymes. For glycolysis, phosphofructokinase and pyruvate kinase were considered to be pacemakers, these being the two glycolytic enzymes for which the reactions are irreversible under physiological conditions. Several metabolites are moreover known to regulate the activities of these enzymes, as noted in the previous paragraph. But changing the amounts of individual enzymes in cells by genetic manipulation has shown that large changes in the activities of phosphofructokinase and pyruvate kinase have little effect on respiration rate. It now appears that all steps in glycolysis and the Krebs cycle contribute to regulation of respiration rate, though not all to the same degree. From the viewpoint of the cell, the larger the number of control points, the more opportunities there are for finetuning and interactions between pathways. From the viewpoint of the investigator, it makes the study of rate control exceedingly complex. There is nevertheless no doubt regarding the basic principle: respiration rate is integrated with cellular activity so that a depletion of respiratory products (ATP and metabolites) leads to an increase in the rate of respiration. An accumulation of the same leads to a decrease in the respiration rate and in each case a new steady state is achieved.

C. NITROGEN METABOLISM: NITRATE AND AMMONIUM ASSIMILATION; BIOLOGICAL NITROGEN FIXATION

Higher plants are autotrophic organisms that can synthesize their organic molecular components out of inorganic nutrients obtained from their surroundings. For many mineral nutrients, this process involves absorption from the soil by the roots and incorporation into the organic compounds that are essential for growth and development. This incorporation of mineral nutrients into organic substances such as pigments, enzyme cofactors, lipids, nucleic acids, and amino acids is termed **nutrient assimilation**.

Assimilation of some nutrients—particularly nitrogen requires a complex series of biochemical reactions that are among the most energy-requiring reactions in living organisms:

- In nitrate (NO_3^-) assimilation, the nitrogen in NO_3^- is converted to a higher-energy form in nitrite (NO_2^-), then to a yet higher-energy form in ammonium (NH_4^+), and finally into the amide nitrogen of glutamine. This process consumes the equivalent of 12 ATPs per nitrogen.
- Plants such as legumes form symbiotic relationships with nitrogen- fixing bacteria to convert molecular nitrogen (N_2) into ammonia (NH_3). Ammonia (NH_3) is the first stable product of natural fixation; at physiological pH, however, ammonia is protonated to form the ammonium ion (NH_4^+). The process of biological nitrogen fixation, together with the subsequent assimilation of NH3 into an amino acid, consumes about 16 ATPs per nitrogen.

For some perspective on the enormous energies involved, consider that if these reactions run rapidly in reverse—say, from NH_4NO_3 (ammonium nitrate) to N_2—they become explosive, liberating vast amounts of energy as motion, heat, and light. Nearly all explosives are based on the rapid oxidation of nitrogen or sulfur compounds.

NITROGEN IN THE ENVIRONMENT

Many biochemical compounds present in plant cells contain nitrogen. For example, nitrogen is found in the nucleoside phosphates and amino acids that form the building blocks of nucleic acids and proteins, respectively. Only the elements oxygen, carbon, and hydrogen are more abundant in plants than nitrogen. Most natural and agricultural ecosystems show dramatic gains in productivity after fertilization with inorganic nitrogen, attesting to the importance of this element. In this section we will discuss the biogeochemical cycle of nitrogen, the crucial role of nitrogen fixation in the conversion of molecular nitrogen into ammonium and nitrate, and the fate of nitrate and ammonium in plant tissues.

Nitrogen Passes through Several Forms in a Biogeochemical Cycle

Nitrogen is present in many forms in the biosphere. The atmosphere contains vast quantities (about 78% by volume) of molecular nitrogen (N_2). For the most part, this large reservoir of nitrogen is not directly available to living organisms. Acquisition of nitrogen from the atmosphere requires the breaking of an exceptionally stable triple covalent bond between two nitrogen atoms (N—N) to produce ammonia (NH_3) or nitrate (NO_3^-).

These reactions, known as **nitrogen fixation,** can be accomplished by both industrial and natural processes. Under elevated temperature (about 200°C) and high pressure (about 200 atmospheres), N_2 combines with hydrogen to form ammonia. The extreme conditions are required to overcome the high activation energy of the reaction. This nitrogen fixation reaction, called the *Haber–Bosch process*, is a starting point for the manufacture of many industrial and agricultural products. Worldwide industrial production of nitrogen fertilizers amounts to more than 80×10^{12} g yr^{-1}. Natural processes fix about 190×10^{12} g yr^{-1} of nitrogen (Table 1) through the following processes:

TABLE 1
The major processes of the biogeochemical nitrogen cycle

Process	Definition	Rate (10^{12} g yr^{-1})[a]
Industrial fixation	Industrial conversion of molecular nitrogen to ammonia	80
Atmospheric fixation	Lightning and photochemical conversion of molecular nitrogen to nitrate	19
Biological fixation	Prokaryotic conversion of molecular nitrogen to ammonia	170
Plant acquisition	Plant absorption and assimilation of ammonium or nitrate	1200
Immobilization	Microbial absorption and assimilation of ammonium or nitrate	N/C
Ammonification	Bacterial and fungal catabolism of soil organic matter to ammonium	N/C
Nitrification	Bacterial (*Nitrosomonas* sp.) oxidation of ammonium to nitrite and subsequent bacterial (*Nitrobacter* sp.) oxidation of nitrite to nitrate	N/C
Mineralization	Bacterial and fungal catabolism of soil organic matter to mineral nitrogen through ammonification or nitrification	N/C
Volatilization	Physical loss of gaseous ammonia to the atmosphere	100
Ammonium fixation	Physical embedding of ammonium into soil particles	10
Denitrification	Bacterial conversion of nitrate to nitrous oxide and molecular nitrogen	210
Nitrate leaching	Physical flow of nitrate dissolved in groundwater out of the topsoil and eventually into the oceans	36

Note: Terrestrial organisms, the soil, and the oceans contain about 5.2×10^{15} g, 95×10^{15} g, and 6.5×10^{15} g, respectively, of organic nitrogen that is active in the cycle. Assuming that the amount of atmospheric N_2 remains constant (inputs = outputs), the *mean residence time* (the average time that a nitrogen molecule remains in organic forms) is about 370 years [(pool size)/(fixation input) = $(5.2 \times 10^{15}$ g + 95×10^{15} g)/(80×10^{12} g yr^{-1} + 19×10^{12} g yr^{-1} + 170×10^{12} g yr^{-1})] [a]N/C, not calculated.

- *Lightning*. Lightning is responsible for about 8% of the nitrogen fixed. Lightning converts water vapor and oxygen

into highly reactive hydroxyl free radicals, free hydrogen atoms, and free oxygen atoms that attack molecular nitrogen (N_2) to form nitric acid (HNO_3). This nitric acid subsequently falls to Earth with rain.

• *Photochemical reactions*. Approximately 2% of the nitrogen fixed derives from photochemical reactions between gaseous nitric oxide (NO) and ozone (O_3) that produce nitric acid (HNO_3).

• *Biological nitrogen fixation*. The remaining 90% results from biological nitrogen fixation, in which bacteria or blue-green algae (cyanobacteria) fix N_2 into ammonium (NH_4^+). From an agricultural standpoint, biological nitrogen fixation is critical because industrial production of nitrogen fertilizers seldom meets agricultural demand.

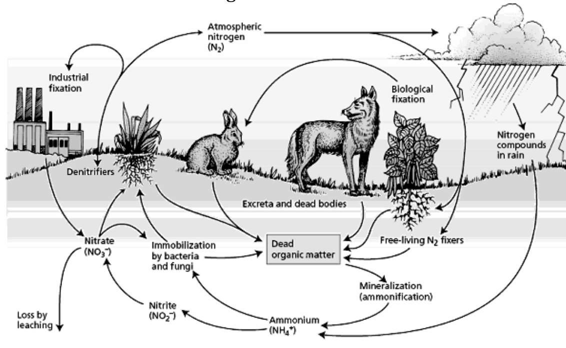


FIGURE 1 Nitrogen cycles through the atmosphere as it changes from a gaseous form to reduced ions before being incorporated into organic compounds in living organisms. Some of the steps involved in the nitrogen cycle are shown.

Once fixed in ammonium or nitrate, nitrogen enters a biogeochemical cycle and passes through several organic or inorganic forms before it eventually returns to molecular nitrogen (Figure 1; see also Table 1). The ammonium (NH_4^+) and nitrate (NO_3^-) ions that are generated through fixation or released through decomposition of soil organic matter become the object of intense competition among plants and microorganisms. To remain competitive, plants have developed mechanisms for scavenging these ions from the soil solution as quickly as possible. Under the elevated soil concentrations that occur after fertilization, the absorption of ammonium and nitrate by the roots may exceed the capacity of a plant to assimilate these ions, leading to their accumulation within the plant's tissues.

Stored Ammonium or Nitrate Can Be Toxic

Plants can store high levels of nitrate, or they can translocate it from tissue to tissue without deleterious effect. However, if livestock and humans consume plant material that is high in nitrate, they may suffer methemoglobinemia, a disease in which the liver reduces nitrate to nitrite, which combines with hemoglobin and renders the hemoglobin unable to bind oxygen. Humans and other animals may also convert nitrate into nitrosamines, which are potent carcinogens. Some countries limit the nitrate content in plant materials sold for human consumption.

In contrast to nitrate, high levels of ammonium are toxic to both plants and animals. Ammonium dissipates transmembrane proton gradients (Figure 2) that are required for both photosynthetic and respiratory electron transport and for sequestering metabolites in the vacuole. Because high levels of ammonium are dangerous, animals have developed a strong aversion to its smell. The active ingredient in smelling salts, a medicinal vapor released under the nose to revive a person who has fainted, is ammonium carbonate. Plants assimilate ammonium near the site of absorption or generation and rapidly store any excess in their vacuoles, thus avoiding toxic effects on membranes and the cytosol.

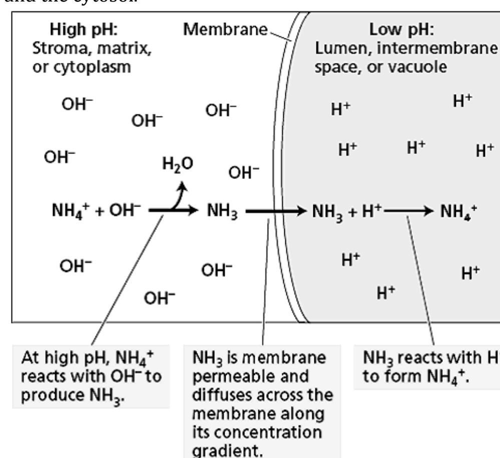


FIGURE 2 NH_4^+ toxicity can dissipate pH gradients. The left side represents the stroma, matrix, or cytoplasm, where the pH is high; the right side represents the lumen, intermembrane space, or vacuole, where the pH is low; and the membrane represents the thylakoid, inner mitochondrial, or tonoplast membrane for a chloroplast, mitochondrion, or root cell, respectively. The net result of the reaction shown is that both the OH^- concentration on the left side and the H^+ concentration on the right side have been diminished; that is, the pH gradient has been dissipated.

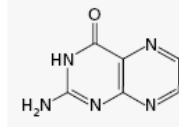
NITRATE ASSIMILATION

Plants assimilate most of the nitrate absorbed by their roots into organic nitrogen compounds. The first step of this process is the reduction of nitrate to nitrite in the cytosol. The enzyme **nitrate reductase** catalyzes this reaction:

$NO_3^- + NAD(P)H + H^+ + 2e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O$ (eq.1)

where NAD(P)H indicates NADH or NADPH. The most common form of nitrate reductase uses only NADH as an electron donor; another form of the enzyme that is found predominantly in nongreen tissues such as roots can use either NADH or NADPH.

The nitrate reductases of higher plants are composed of two identical subunits, each containing three prosthetic groups: FAD (flavin adenine dinucleotide), heme, and a molybdenum complexed to an organic molecule called a *pterin*.



A pterin (fully oxidized)

Nitrate reductase is the main molybdenum-containing protein in vegetative tissues, and one symptom of molybdenum deficiency is the accumulation of nitrate that results from diminished nitrate reductase activity. Comparison of the amino acid sequences for nitrate reductase from several species with those of other wellcharacterized proteins that bind FAD, heme, or molybdenum has led to the three-domain model for nitrate reductase shown in Figure 3. The FAD-binding domain accepts two electrons from NADH or NADPH. The electrons then pass through the heme domain to the molybdenum complex, where they are transferred to nitrate.

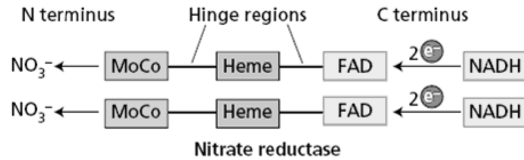


FIGURE 3 A model of the nitrate reductase dimer, illustrating the three binding domains whose polypeptide sequences are similar in eukaryotes: molybdenum complex (MoCo), heme, and FAD. The NADH binds at the FAD-binding region of each subunit and initiates a two-electron transfer from the carboxyl (C) terminus, through each of the electron transfer components, to the amino (N) terminus. Nitrate is reduced at the molybdenum complex near the amino terminus. The polypeptide sequences of the hinge regions are highly variable among species.

Nitrate, Light, and Carbohydrates Regulate Nitrate Reductase

Nitrate, light, and carbohydrates influence nitrate reductase at the transcription and translation levels. In barley seedlings, nitrate reductase mRNA was detected approximately 40 minutes after addition of nitrate, and maximum levels were attained within 3 hours (Figure 4). In contrast to the rapid mRNA accumulation, here was a gradual linear increase in nitrate reductase activity, reflecting the slower synthesis of the protein. In addition, the protein is subject to posttranslational modulation (involving a reversible phosphorylation) that is analogous to the regulation of sucrose phosphate synthase. Light, carbohydrate levels, and other environmental factors stimulate a protein phosphatase that dephosphorylates several serine residues on the nitrate reductase protein and thereby activates the enzyme.

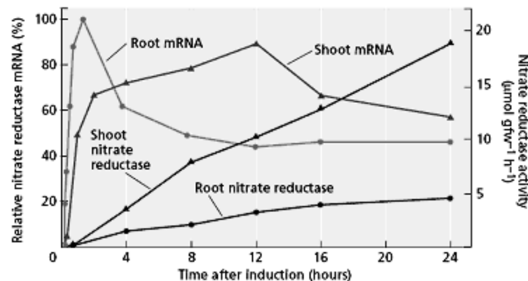
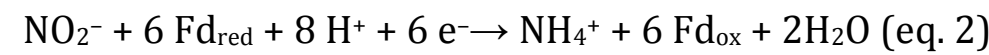


FIGURE 4 Stimulation of nitrate reductase activity follows the induction of nitrate reductase mRNA in shoots and roots of barley; gfw, grams fresh weight.

Operating in the reverse direction, darkness and Mg^{2+} stimulate a protein kinase that phosphorylates the same serine residues, which then interact with a 14-3-3 inhibitor protein, and thereby inactivate nitrate reductase. *Regulation of nitrate reductase activity through phosphorylationand dephosphorylation provides more rapid control than can be achieved through synthesis or degradation of theenzyme (minutes versus hours).*

Nitrite Reductase Converts Nitrite to Ammonium

Nitrite (NO_2^-) is a highly reactive, potentially toxic ion. Plant cells immediately transport the nitrite generated by nitrate reduction from the cytosol into chloroplasts in leaves and plastids in roots. In these organelles, the enzyme nitrite reductase reduces nitrite to ammonium according to the following overall reaction:



$$NO_2^- + 6\ Fd_{red} + 8\ H^+ + 6\ e^- \rightarrow NH_4^+ + 6\ Fd_{ox} + 2H_2O \quad \text{(eq. 2)}$$

where Fd is ferredoxin, and the subscripts *red* and *ox* stand for *reduced* and *oxidized*, respectively. Reduced ferredoxin derives from photosynthetic electron transport in the chloroplasts and from NADPH generated by the oxidative pentose phosphate pathway in nongreen tissues. Chloroplasts and root plastids contain different forms of the enzyme, but both forms consist of a single polypeptide containing two prosthetic groups: an iron–sulfur cluster (Fe_4S_4) and a specialized heme.

These groups acting together bind nitrite and reduce it directly to ammonium, without accumulation of nitrogen compounds of intermediate redox states. The electron flow through ferredoxin (Fe_4S_4) and heme can be represented as in Figure 5.

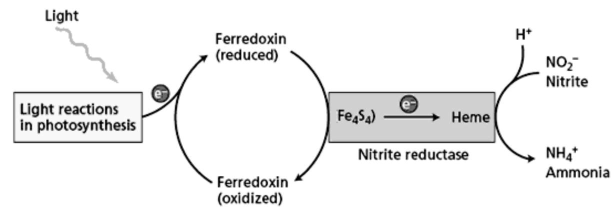


FIGURE 5 Model for coupling of photosynthetic electron flow, via ferredoxin, to the reduction of nitrite by nitrite reductase. The enzyme contains two prosthetic groups, Fe_4S_4 and heme, which participate in the reduction of nitrite to ammonium.

Nitrite reductase is encoded in the nucleus and synthesized in the cytoplasm with an N-terminal transit peptide that targets it to the plastids. Whereas NO_3^- and light induce the transcription of nitrite reductase mRNA, the end products of the process—asparagine and glutamine—repress this induction.

Plants Can Assimilate Nitrate in Both Roots and Shoots

In many plants, when the roots receive small amounts of nitrate, nitrate is reduced primarily in the roots. As the supply of nitrate increases, a greater proportion of the absorbed nitrate is translocated to the shoot and assimilated there. Even under similar conditions of nitrate supply, the balance between root and shoot nitrate metabolism—as indicated by the proportion of nitrate reductase activity in each of the two organs or by the relative concentrations of nitrate and reduced nitrogen in the xylem sap—varies from species to species.

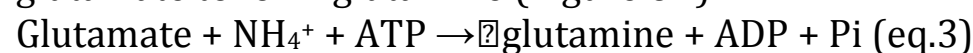
In plants such as the cocklebur (*Xanthium strumarium*), nitrate metabolism is restricted to the shoot; in other plants, such as white lupine (*Lupinus albus*), most nitrate is metabolized in the roots. Generally, species native to temperate regions rely more heavily on nitrate assimilation by the roots than do species of tropical or subtropical origins.

AMMONIUM ASSIMILATION

Plant cells avoid ammonium toxicity by rapidly converting the ammonium generated from nitrate assimilation or photorespiration into amino acids. The primary pathway for this conversion involves the sequential actions of glutamine synthetase and glutamate synthase. In this section we will discuss the enzymatic processes that mediate the assimilation of ammonium into essential amino acids, and the role of amides in the regulation of nitrogen and carbon metabolism.

Conversion of Ammonium to Amino Acids Requires Two Enzymes

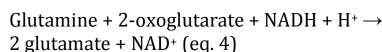
Glutamine synthetase (**GS**) combines ammonium with glutamate to form glutamine (Figure 6A):



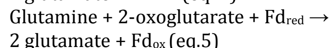
Glutamate + NH_4^+ + ATP → glutamine + ADP + Pi (eq.3)

This reaction requires the hydrolysis of one ATP and involves a divalent cation such as Mg^{2+}, Mn^{2+}, or Co^{2+} as a cofactor. Plants contain two classes of GS, one in the cytosol and the other in root plastids or shoot chloroplasts. The cytosolic forms are expressed in germinating seeds or in the vascular bundles of roots and shoots and produce glutamine for intracellular nitrogen transport. The GS in root plastids generates amide nitrogen for local consumption; the GS in shoot chloroplasts reassimilates photorespiratory NH_4^+. Light and carbohydrate levels alter
the expression of the plastid forms of the enzyme, but they have little effect on the cytosolic forms.

Elevated plastid levels of glutamine stimulate the activity of **glutamate synthase** (also known as *glutamine:2-oxoglutarate aminotransferase*, or **GOGAT**). This enzyme transfers the amide group of glutamine to 2-oxoglutarate, yielding two molecules of glutamate (see Figure 6A). Plants contain two types of GOGAT: One accepts electrons from NADH; the other accepts electrons from ferredoxin (Fd):



Glutamine + 2-oxoglutarate + NADH + H^+ →
2 glutamate + NAD^+ (eq. 4)



Glutamine + 2-oxoglutarate + Fd_{red} →
2 glutamate + Fd_{ox} (eq.5)

The NADH type of the enzyme (NADH-GOGAT) is located in plastids of nonphotosynthetic tissues such as roots or vascular bundles of developing leaves. In roots, NADH-GOGAT is involved in the assimilation of NH_4^+ absorbed from the rhizosphere (the soil near the surface of the roots); in vascular bundles of developing leaves, NADH-GOGAT assimilates glutamine translocated from roots or senescing leaves.

The ferredoxin-dependent type of glutamate synthase (Fd-GOGAT) is found in chloroplasts and serves in photorespiratory nitrogen metabolism. Both the amount of protein and its activity increase with light levels. Roots, particularly those under nitrate nutrition, have Fd-GOGAT in plastids. Fd-GOGAT in the roots presumably functions to incorporate the glutamine generated during nitrate assimilation.

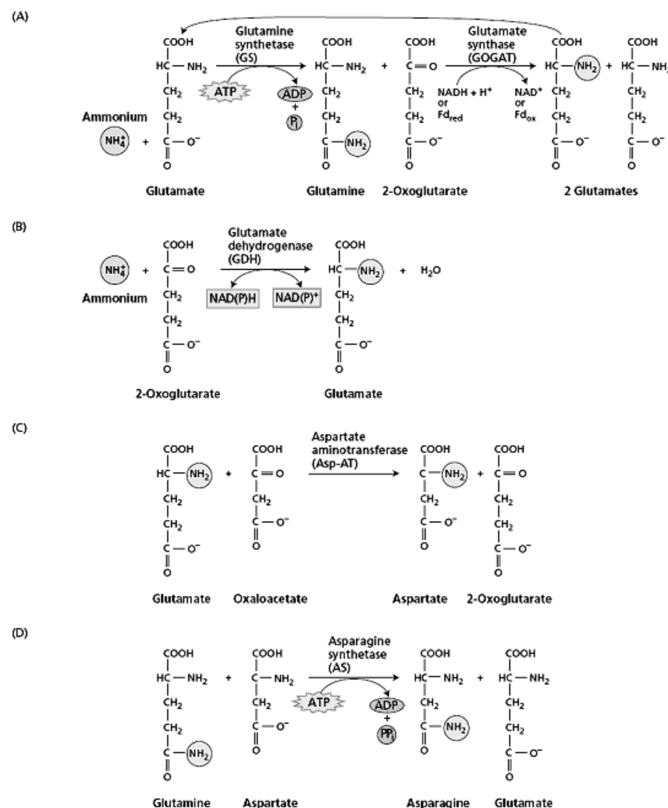
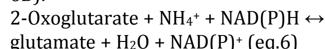


FIGURE 7 Structure and pathways of compounds involved in ammonium metabolism. Ammonium can be assimilated by one of several processes. (A) The GS-GOGAT pathway that forms glutamine and glutamate. A reduced cofactor is required for the reaction: ferredoxin in green leaves and NADH in nonphotosynthetic tissue. (B) The GDH pathway that forms glutamate using NADH or NADPH as a reductant. (C) Transfer of the amino group from glutamate to oxaloacetate to form aspartate (catalyzed by aspartate aminotransferase). (D) Synthesis of asparagine by transfer of an amino acid group from glutamine to aspartate (catalyzed by asparagine synthesis).

Ammonium Can Be Assimilated via an Alternative Pathway

Glutamate dehydrogenase (**GDH**) catalyzes a reversible reaction that synthesizes or deaminates glutamate (Figure 6B):

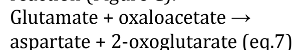


2-Oxoglutarate + NH_4^+ + NAD(P)H ↔
glutamate + H_2O + $NAD(P)^+$ (eq.6)

An NADH-dependent form of GDH is found in mitochondria, and an NADPH-dependent form is localized in the chloroplasts of photosynthetic organs. Although both forms are relatively abundant, they cannot substitute for the GS–GOGAT pathway for assimilation of ammonium, and their primary function is to deaminate glutamate (see Figure 6B).

Transamination Reactions Transfer Nitrogen

Once assimilated into glutamine and glutamate, nitrogen is incorporated into other amino acids via transamination reactions. The enzymes that catalyze these reactions are known as aminotransferases. An example is **aspartate aminotransferase** (**Asp-AT**), which catalyzes the following reaction (Figure C):

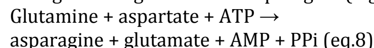


Glutamate + oxaloacetate →
aspartate + 2-oxoglutarate (eq.7)

in which the amino group of glutamate is transferred to the carboxyl atom of aspartate. Aspartate is an amino acid that participates in the malate–aspartate shuttle to transfer reducing equivalents from the mitochondrion and chloroplast into the cytosol and in the transport of carbon from mesophyll to bundle sheath for C4 carbon fixation. All transamination reactions require pyridoxal phosphate (vitamin B6) as a cofactor. Aminotransferases are found in the cytoplasm, chloroplasts, mitochondria, glyoxysomes, and peroxisomes. The aminotransferases localized in the chloroplasts may have a significant role in amino acid biosynthesis because plant leaves or isolated chloroplasts exposed to radioactively labeled carbon dioxide rapidly incorporate the label into glutamate, aspartate, alanine, serine, and glycine.

Asparagine and Glutamine Link Carbon and Nitrogen Metabolism

Asparagine, isolated from asparagus as early as 1806, was the first amide to be identified. It serves not only as a protein precursor, but as a key compound for nitrogen transport and storage because of its stability and high nitrogen-to-carbon ratio (2 N to 4 C for asparagine, versus 2 N to 5 C for glutamine or 1 N to 5 C for glutamate). The major pathway for asparagine synthesis involves the transfer of the amide nitrogen from glutamine to asparagine (Figure 6D):



Glutamine + aspartate + ATP →
asparagine + glutamate + AMP + PPi (eq.8)

Asparagine synthetase (**AS**), the enzyme that catalyzes this reaction, is found in the cytosol of leaves and roots and in nitrogen-fixing nodules (see the next . In maize roots, particularly those under potentially toxic levels of ammonia, ammonium may replace glutamine as the source of the amide group.

High levels of light and carbohydrate—conditions that stimulate plastid GS and Fd-GOGAT—inhibit the expression of genes coding for AS and the activity of the enzyme. The opposing regulation of these competing pathways helps balance the metabolism of carbon and nitrogen in plants. Conditions of ample energy (i.e., high levels of light and carbohydrates) stimulate GS and GOGAT, inhibit AS, and thus favor nitrogen assimilation into glutamine and glutamate, compounds that are rich in carbon and participate in the synthesis of new plant materials.

By contrast, energy-limited conditions inhibit GS and GOGAT, stimulate AS, and thus favor nitrogen assimilation into asparagine, a compound that is rich in nitrogen and sufficiently stable for long-distance transport or long-term storage.

BIOLOGICAL NITROGEN FIXATION

Biological nitrogen fixation accounts for most of the fixation of atmospheric N_2 into ammonium, thus representing the key entry point of molecular nitrogen into the biogeochemical cycle of nitrogen (see Figure 1). This section describes the properties of the nitrogenase enzymes that fix nitrogen, the symbiotic relations between nitrogen-fixing organisms and higher plants, the specialized structures that form in roots when infected by nitrogen-fixing bacteria, and the genetic and signaling interactions that regulate nitrogen fixation by symbiotic prokaryotes and their hosts.

Free-Living and Symbiotic Bacteria Fix Nitrogen

Some bacteria, as stated earlier, can convert atmospheric nitrogen into ammonium (Table 2). Most of these nitrogen-fixing prokaryotes are free-living in the soil. A few form symbiotic associations with higher plants in which the prokaryote directly provides the host plant with fixed nitrogen in exchange for other nutrients and carbohydrates (top portion of Table 2). Such symbioses occur in nodules that form on the roots of the plant and contain the nitrogen-fixing bacteria. The most common type of symbiosis occurs between members of the plant family Leguminosae and soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Photorhizobium, Rhizobium*, and *Sinorhizobium* (collectively called **rhizobia**; Table 3). Another common type of symbiosis occurs between several woody plant species, such as alder trees, and soil bacteria of the genus *Frankia*. Still other types involve the South American herb *Gunnera* and the tiny water fern *Azolla*, which form associations with the cyanobacteria *Nostoc* and *Anabaena*, respectively (see Table 2).

TABLE 2
Examples of organisms that can carry out nitrogen fixation

Symbiotic nitrogen fixation	
Host plant	**N-fixing symbionts**
Leguminous: legumes, *Parasponia*	*Azorhizobium, Bradyrhizobium, Photorhizobium, Rhizobium, Sinorhizobium*
Actinorhizal: alder (tree), *Ceanothus* (shrub), *Casuarina* (tree), *Datisca* (shrub)	*Frankia*
Gunnera	*Nostoc*
Azolla (water fern)	*Anabaena*
Sugarcane	*Acetobacter*
Free-living nitrogen fixation	
Type	**N-fixing genera**
Cyanobacteria (blue-green algae)	*Anabaena, Calothrix, Nostoc*
Other bacteria	
Aerobic	*Azospirillum, Azotobacter, Beijerinckia, Derxia*
Facultative	*Bacillus, Klebsiella*
Anaerobic	
Nonphotosynthetic	*Clostridium, Methanococcus* (archaebacterium)
Photosynthetic	*Chromatium, Rhodospirillum*

Nitrogen Fixation Requires Anaerobic Conditions

Because oxygen irreversibly inactivates the **nitrogenase** enzymes involved in nitrogen fixation, nitrogen must be fixed under anaerobic conditions. Thus each of the nitrogen-fixing organisms listed in Table 2 either functions under natural anaerobic conditions or can create an internal anaerobic environment in the presence of oxygen. In cyanobacteria, anaerobic conditions are created in specialized cells called *heterocysts*. Heterocysts are thick-walled cells that differentiate when filamentous cyanobacteria are deprived of NH_4^+. These cells lack photosystem II, the oxygen-producing photosystem of chloroplasts, so they do not generate oxygen. Heterocysts appear to represent an adaptation for nitrogen fixation, in that they are widespread among aerobic cyanobacteria that fix nitrogen.

Cyanobacteria that lack heterocysts can fix nitrogen only under anaerobic conditions such as those that occur in flooded fields. In Asian countries, nitrogen-fixing cyanobacteria of both the heterocyst and nonheterocyst types are a major means for maintaining an adequate nitrogen supply in the soil of rice fields. These microorganisms fix nitrogen when the fields are flooded and die as the fields dry, releasing the fixed nitrogen to the soil. Another important ource of available nitrogen in flooded rice fields is the water fern *Azolla*, which associates with the cyanobacterium *Anabaena*. The *Azolla–Anabaena* association can fix as much as 0.5 kg of atmospheric nitrogen per hectare per day, a rate of fertilization that is sufficient to attain moderate rice yields.

Free-living bacteria that are capable of fixing nitrogen are aerobic, facultative, or anaerobic (see Table 2, bottom):

• *Aerobic* nitrogen-fixing bacteria such as *Azotobacter* are thought to maintain reduced oxygen conditions (microaerobic conditions) through their high levels of respiration. Others, such as *Gloeothece*, evolve O_2 photosynthetically during the day and fix nitrogen during the night.

• *Facultative* organisms, which are able to grow under both aerobic and anaerobic conditions, generally fix nitrogen only under anaerobic conditions.

• For *anaerobic* nitrogen-fixing bacteria, oxygen does not pose a problem, because it is absent in their habitat.

These anaerobic organisms can be either photosynthetic (e.g., *Rhodospirillum*), or nonphotosynthetic (e.g., *Clostridium*).

Symbiotic Nitrogen Fixation Occurs in Specialized Structures

Symbiotic nitrogen-fixing prokaryotes dwell within **nodules**, the special organs of the plant host that enclose the nitrogen-fixing bacteria. In the case of *Gunnera*, these organs are existing stem glands that develop independently of the symbiont. In the case of legumes and actinorhizal plants, the nitrogen-fixing bacteria induce the plant to form root nodules.

Grasses can also develop symbiotic relationships with nitrogen-fixing organisms, but in these associations root nodules are not produced. Instead, the nitrogen-fixing bacteria seem to colonize plant tissues or anchor to the root surfaces, mainly around the elongation zone and the root hairs. For example, the nitrogen-fixing bacterium *Acetobacter diazotrophicus* lives in the apoplast of stem tissues in sugarcane and may provide its host with sufficient nitrogen to grant independence from nitrogen fertilization. The potential for applying *Azospirillum* to corn and other grains has been explored, but *Azospirillum* seems to fix little nitrogen when associated with plants.

Legumes and actinorhizal plants regulate gas permeability in their nodules, maintaining a level of oxygen within the nodule that can support respiration but is sufficiently low to avoid inactivation of the nitrogenase. Gas permeability increases in the light and decreases under drought or upon exposure to nitrate. The mechanism for regulating gas permeability is not yet known.

TABLE 3 Associations between host plants and rhizobia

Plant host	Rhizobial symbiont
Parasponia (a nonlegume, formerly called *Trema*)	*Bradyrhizobium* spp.
Soybean (*Glycine max*)	*Bradyrhizobium japonicum* (slow-growing type); *Sinorhizobium fredii* (fast-growing type)
Alfalfa (*Medicago sativa*)	*Sinorhizobium meliloti*
Sesbania (aquatic)	*Azorhizobium* (forms both root and stem nodules; the stems have adventitious roots)
Bean (*Phaseolus*)	*Rhizobium leguminosarum* bv. *phaseoli*; *Rhizobium tropicii*; *Rhizobium etli*
Clover (*Trifolium*)	*Rhizobium leguminosarum* bv. *trifolii*
Pea (*Pisum sativum*)	*Rhizobium leguminosarum* bv. *viciae*
Aeschenomene (aquatic)	*Photorhizobium* (photosynthetically active rhizobia that form stem nodules, probably associated with adventitious roots)

Nodules contain an oxygen-binding heme protein called **leghemoglobin**. Leghemoglobin is present in the cytoplasm of infected nodule cells at high concentrations (700 μM in soybean nodules) and gives the nodules a pink color.

The host plant produces the globin portion of leghemoglobin in response to infection by the bacteria; the bacterial symbiont produces the heme portion. Leghemoglobin has a high affinity for oxygen (a *K*m of about 0.01 μ*M*), about ten times higher than the β chain of human hemoglobin.

Although leghemoglobin was once thought to provide a buffer for nodule oxygen, recent studies indicate that it stores only enough oxygen to support nodule respiration for a few seconds. Its function is to help transport oxygen to the respiring symbiotic bacterial cells in a manner analogous to hemoglobin transporting oxygen to respiring tissues in animals.

Establishing Symbiosis Requires an Exchange of Signals

The symbiosis between legumes and rhizobia is not obligatory. Legume seedlings germinate without any association with rhizobia, and they may remain unassociated throughout their life cycle. Rhizobia also occur as free-living organisms in the soil. Under nitrogen-limited conditions, however, the symbionts seek out one another through an elaborate exchange of signals. This signaling, the subsequent infection process, and the development of nitrogenfixing nodules involve specific genes in both the host and the symbionts.

Plant genes specific to nodules are called **nodulin** (*Nod*) genes; rhizobial genes that participate in nodule formation are called **nodulation** (*nod*) genes. The *nod* genes are classified as common *nod* genes or host-specific *nod* genes. The common *nod* genes—*nodA*, *nodB*, and *nodC*—are found in all rhizobial strains; the host-specific *nod* genes—such as *nodP*, *nodQ*, and *nodH*; or *nodF*, *nodE*, and *nodL*—differ

among rhizobial species and determine the host range. Only one of the *nod* genes, the regulatory *nodD*, is constitutively expressed, and as we will explain in detail, its protein product (NodD) regulates the transcription of the other *nod* genes.

The first stage in the formation of the symbiotic relationship between the nitrogen-fixing bacteria and their host is migration of the bacteria toward the roots of the host plant. This migration is a chemotactic response mediated by chemical attractants, especially (iso)flavonoids and betaines, secreted by the roots. These attractants activate the rhizobial NodD protein, which then induces transcription of the other *nod* genes.

The promoter region of all *nod* operons, except that of *nodD*, contains a highly conserved sequence called the *nod* box. Binding of the activated NodD to the *nod* box induces transcription of the other *nod* genes.

Nod Factors Produced by Bacteria Act as Signals for Symbiosis

The *nod* genes activated by NodD code for nodulation proteins, most of which are involved in the biosynthesis of Nod factors. **Nod factors** are lipochitin oligosaccharide signal molecules, all of which have a chitin β-1→4-linked *N*acetyl- D-glucosamine backbone (varying in length from three to six sugar units) and a fatty acyl chain on the C-2 position of the nonreducing sugar (Figure 7).

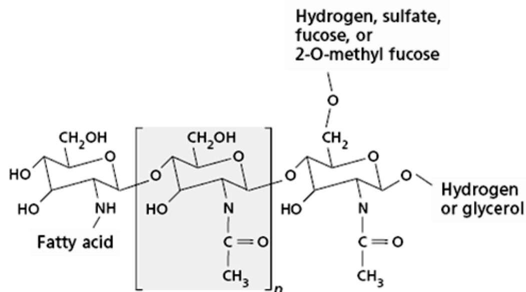


FIGURE 7 Nod factors are lipochitin oligosaccharides. The fatty acid chain typically has 16 to 18 carbons. The number of repeated middle sections (*n*) is usually 2 to 3.

Three of the *nod* genes (*nodA*, *nodB*, and *nodC*) encode enzymes (NodA, NodB, and NodC, respectively) that are required for synthesizing this basic structure:

1. NodA is an *N*-acyltransferase that catalyzes the addition of a fatty acyl chain.
2. NodB is a chitin-oligosaccharide deacetylase that removes the acetyl group from the terminal nonreducing sugar.
3. NodC is a chitin-oligosaccharide synthase that links *N*-acetyl-D-glucosamine monomers.

Host-specific *nod* genes that vary among rhizobial species are involved in the modification of the fatty acyl chain or the addition of groups important in determining host specificity:

• NodE and NodF determine the length and degree of saturation of the fatty acyl chain; those of *Rhizobium leguminosarum* bv. *viciae* and *R. meliloti* result in the synthesis of an 18:4 and a 16:2 fatty acyl group, respectively.
• Other enzymes, such as NodL, influence the host specificity of Nod factors through the addition of specific substitutions at the reducing or nonreducing sugar moieties of the chitin backbone.

A particular legume host responds to a specific Nod factor.The legume receptors for Nod factors appear to be special lectins (sugar-binding proteins) produced in the root hairs. Nod factors activate these lectins, increasing their hydrolysis of phosphoanhydride bonds of nucleoside di- and triphosphates. This lectin activation directs particular rhizobia to appropriate hosts and facilitates attachment of the rhizobia to the cell walls of a root hair.

Nodule Formation Involves Several Phytohormones

Two processes—infection and nodule organogenesis—occur simultaneously during root nodule formation. During the infection process, rhizobia that are attached to the root hairs release Nod factors that induce a pronounced curling of the root hair cells (Figure 8A and B). The rhizobia become enclosed in the small compartment formed by the curling. The cell wall of the root hair degrades in these regions, also in response to Nod factors, allowing the bacterial cells direct access to the outer surface of the plant plasma membrane.

The next step is formation of the **infection thread** (Figure 8C), an internal tubular extension of the plasma membrane that is produced by the fusion of Golgi-derived membrane vesicles at the site of infection. The thread grows at its tip by the fusion of secretory vesicles to the end of the tube. Deeper into the root cortex, near the xylem, cortical cells dedifferentiate and start dividing, forming a distinct area within the cortex, called a *nodule primordium*, from which the nodule will develop. The nodule primordia form opposite the protoxylem poles of the root vascular bundle.

Different signaling compounds, acting either positively or negatively, control the position of nodule primordia. The nucleoside uridine diffuses from the stele into the cortex in the protoxylem zones of the root and stimulates cell division. Ethylene is synthesized in the region of the pericycle, diffuses into the cortex, and blocks cell division opposite the phloem poles of the root. The infection thread filled with proliferating rhizobia elongates through the root hair and cortical cell layers, in the direction of the nodule primordium. When the infection thread reaches specialized cells within the nodule, its tip fuses with the plasma membrane of the host cell, releasing bacterial cells that are packaged in a membrane derived from the host cell plasma membrane (see Figure 8D).

Branching of the infection thread inside the nodule enables the bacteria to infect many cells (see Figure 8E and F). At first the bacteria continue to divide, and the surrounding membrane increases in surface area to accommodate this growth by fusing with smaller vesicles. Soon thereafter, upon an undetermined signal from the plant, the bacteria stop dividing and begin to enlarge and to differentiate into nitrogen-fixing endosymbiotic organelles called **bacteroids**. The membrane surrounding the bacteroids is called the *peribacteroid membrane*.

The nodule as a whole develops such features as a vascular system (which facilitates the exchange of fixed nitrogen produced by the bacteroids for nutrients contributed by the plant) and a layer of cells to exclude O_2 from the root nodule interior. In some temperate legumes (e.g., peas), the nodules are elongated and cylindrical because of the presence of a *nodule meristem*. The nodules of tropical legumes, such as soybeans and peanuts, lack a persistent meristem and are spherical.

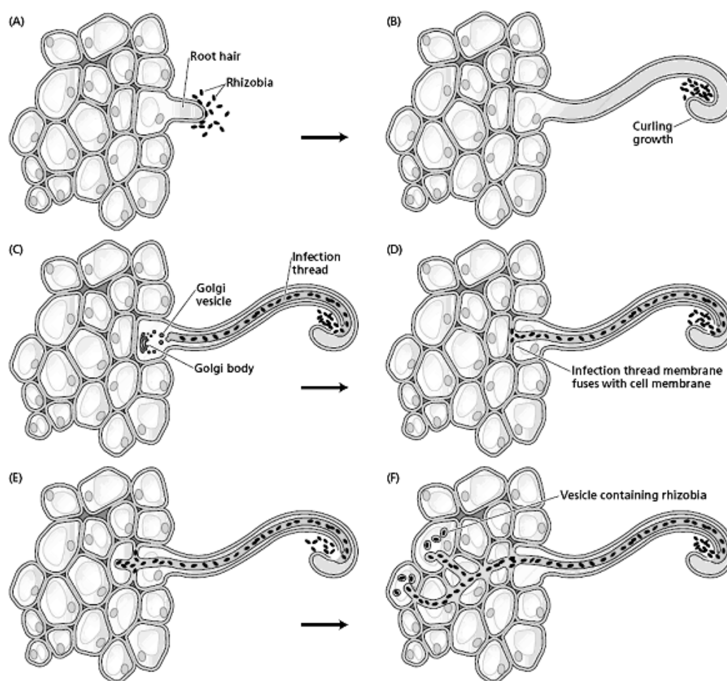
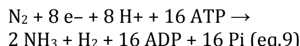


FIGURE 8 The infection process during nodule organogenesis.
(A) Rhizobia bind to an emerging root hair in response to chemical attractants sent by the plant. (B) In response to factors produced by the bacteria, the root hair exhibits abnormal curling growth, and rhizobia cells proliferate within the coils. (C) Localized degradation of the root hair wall leads to infection and formation of the infection thread from Golgi secretory vesicles of root cells. (D) The infection thread reaches the end of the cell, and its membrane fuses with the plasma membrane of the root hair cell. (E) Rhizobia are released into the apoplast and penetrate the compound middle lamella to the subepidermal cell plasma membrane, leading to the initiation of a new infection thread, which forms an open channel with the first. (F) The infection thread extends and branches until it reaches target cells, where vesicles composed of plant membrane that enclose bacterial cells are released into the cytosol.

The Nitrogenase Enzyme Complex Fixes N_2

Biological nitrogen fixation, like industrial nitrogen fixation, produces ammonia from molecular nitrogen. The overall reaction is

$N_2 + 8 e^- + 8 H^+ + 16 ATP \rightarrow$


$2 NH_3 + H_2 + 16 ADP + 16 Pi$ (eq.9)

Note that the reduction of N_2 to 2 NH_3, a six-electron transfer, is coupled to the reduction of two protons to evolve H_2. The **nitrogenase enzyme complex** catalyzes this reaction. The nitrogenase enzyme complex can be separated into two components—the Fe protein and the MoFe protein— neither of which has catalytic activity by itself (Figure 9):

• The Fe protein is the smaller of the two components and has two identical subunits of 30 to 72 kDa each, depending on the organism. Each subunit contains an iron–sulfur cluster (4 Fe and 4 S^{2-}) that participates in the redox reactions involved in the conversion of N_2 to NH_3. The Fe protein is irreversibly inactivated by O_2 with typical half-decay times of 30 to 45 seconds.

• The MoFe protein has four subunits, with a total molecular mass of 180 to 235 kDa, depending on the species. Each subunit has two Mo–Fe–S clusters. The MoFe protein is also inactivated by oxygen, with a half-decay time in air of 10 minutes.

In the overall nitrogen reduction reaction (see Figure 9), ferredoxin serves as an electron donor to the Fe protein, which in turn hydrolyzes ATP and reduces the MoFe protein. The MoFe protein then can reduce numerous substrates (Table 4), although under natural conditions it reacts only with N_2 and H^+. One of the reactions catalyzed by nitrogenase, the reduction of acetylene to ethylene, is used in estimating nitrogenase activity.

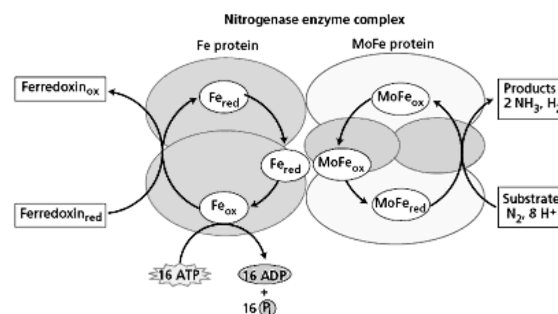


FIGURE 9 The reaction catalyzed by nitrogenase. Ferredoxin reduces the Fe protein. Binding and hydrolysis of ATP to the Fe protein is thought to cause a conformational change of the Fe protein that facilitates the redox reactions. The Fe protein reduces the MoFe protein, and the MoFe protein reduces the N2.

The energetics of nitrogen fixation is complex. The production of NH_3 from N_2 and H_2 is an exergonic reaction, with a $\Delta G^{0`}$(change in free energy) of –27 kJ mol^{-1}. However, industrial production of NH_3 from N_2 and H_2 is *endergonic*, requiring a very large energy input because of the activation energy needed to break the triple bond in N_2. For the same reason, the enzymatic reduction of N_2 by nitrogenase also requires a large investment of energy (see Equation 9), although the exact changes in free energy are not yet known.

Calculations based on the carbohydrate metabolism of legumes show that a plant consumes 12 g of organic carbon per gram of N_2 fixed. On the basis of Equation, the $\Delta G^{0`}$ for the overall reaction of biological nitrogen fixation is about –200 kJ mol^{-1}. Because the overall reaction is highly exergonic, ammonium production is limited by the slow operation (number of N_2 molecules reduced per unit time) of the nitrogenase complex.

Under natural conditions, substantial amounts of H^+ are reduced to H_2 gas, and this process can compete with N_2 reduction for electrons from nitrogenase. In rhizobia, 30 to 60% of the energy supplied to nitrogenase may be lost as H_2, diminishing the efficiency of nitrogen fixation. Some rhizobia, however, contain hydrogenase, an enzyme that can split the H_2 formed and generate electrons for N_2 reduction, thus improving the efficiency of nitrogen fixation.

TABLE 4 Reactions catalyzed by nitrogenase

Reaction	
$N_2 \rightarrow NH_3$	Molecular nitrogen fixation
$N_2O \rightarrow N_2 + H_2O$	Nitrous oxide reduction
$N_3^- \rightarrow N_2 + NH_3$	Azide reduction
$C_2H_2 \rightarrow C_2H_4$	Acetylene reduction
$2\ H^+ \rightarrow H_2$	H_2 production
$ATP \rightarrow ADP + P_i$	ATP hydrolytic activity

Amides and Ureides Are the Transported Forms of Nitrogen

The symbiotic nitrogen-fixing prokaryotes release ammonia that, to avoid toxicity, must be rapidly converted into organic forms in the root nodules before being transported to the shoot via the xylem. Nitrogen-fixing legumes can be divided into amide exporters or ureide exporters on the basis of the composition of the xylem sap. Amides (principally the amino acids asparagine or glutamine) are exported by temperate-region legumes, such as pea (*Pisum*), clover (*Trifolium*), broad bean (*Vicia*), and lentil (*Lens*). Ureides are exported by legumes of tropical origin, such as soybean (*Glycine*), kidney bean (*Phaseolus*), peanut (*Arachis*), and southern pea (*Vigna*). The three major ureides are allantoin, allantoic acid, and citrulline.

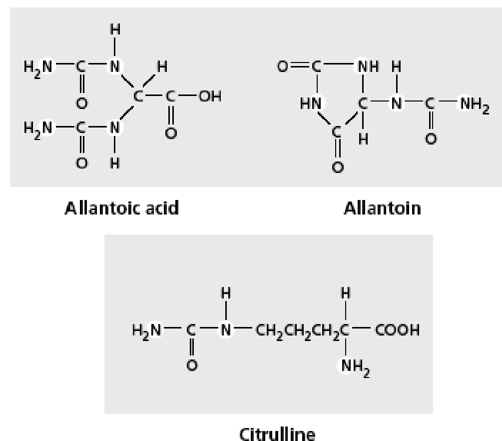


FIGURE The major ureide compounds used to trans port nitrogen from sites of fixation to sites where their deamination will provide nitrogen for amino acid and nucleoside synthesis.

Allantoin is synthesized in peroxisomes from uric acid, and allantoic acid is synthesized from allantoin in the endoplasmic reticulum. The site of citrulline synthesis from the amino acid ornithine has not yet been determined. All three compounds are ultimately released into the xylem and transported to the shoot, where they are rapidly catabolized to ammonium. This ammonium enters the assimilation pathway described earlier.

D. PLANT HORMONES: BIOSYNTHESIS, STORAGE, BREAKDOWN AND TRANSPORT; PHYSIOLOGICAL EFFECTS AND MECHANISMS OF ACTION.

1 Introduction

A constant theme underlying the study of plant physiology is that plant growth and development are controlled by the environment. Plants being sessile organisms, it is not surprising that their development is exquisitely sensitive to a wide range of environmental factors and is extremely plastic, i.e. very flexible. There are underlying basic patterns in plant development, but there is considerable regulation by environmental signals of how and when these patterns are expressed. In addition, there are internal signals within the plant. One of the most important factors influencing the development of a cell is its position within the plant. A plant cell develops depending on its location in relation to neighbouring cells, and this in turn will determine its response to environmental signals. For example, the response to drought of a cell within the leaf will differ in many ways from that of a cell within the root. The key question arises of how a complex set of environmental factors can interact with cells to elicit an appropriate response within a given cell type: what are the internal signals that communicate between cells, and mediate between environmental factors and the plant tissues?

It has been known for decades (if not centuries) that plants contain a range of compounds which have profound effects on many aspects of growth and developmental physiology, and act as a means of communication within the plant. These plant growth hormones, sometimes referred to as plant growth regulators, are still being discovered. They are of supreme importance in controlling cell division, growth and differentiation, which in turn determine the morphology and ultimately the physiology of the whole plant. The concentrations of the hormones are greatly influenced by environmental factors, and the movement of hormones in the plant can also be under environmental control. Although the mechanisms by which they act cannot be considered to be well understood, we are beginning to unravel their modes of action and the ways in which they mutually interact to regulate plant functions.

2 Plant growth hormones

2 .1 Concepts and definitions

In any area of biology, attempting to formulate definitions is difficult and this is especially true of plant growth hormones. One reasonable definition might be: *A plant growth hormone is a substance produced within the plant in very low concentrations and transported to another part of the plant where it causes a response.*

There are a number of key concepts in this definition. Firstly, plant growth hormones are naturally occurring compounds synthesized within the plant. However, similar if not identical compounds can be synthesized by other organisms. For example, many plant pathogens produce substances similar to plant hormones which are important in the progression of the disease. Man-made plant hormones are commercially extremely important as they have such a diverse range of effects on plant development from acting as weedkillers to stimulating fruit development.

The second key concept is that of transport. Plant growth hormones are synthesized throughout the plant but apical meristems and young, developing tissues are rich sources of these compounds. They are transported, perhaps through only a few cells, but often throughout the plant, giving rise to a response in target tissues. It is possible, and has been proposed, that volatile chemical signals might be exchanged between plants although the importance of this in field conditions remains controversial. Thirdly, these compounds cause a response. Different cells will respond in different ways (or not at all) to a particular hormone, giving rise to the concept of 'competence to respond' – that is whether a particular cell type will respond to a given concentration of a plant growth regulator or not.

Fourthly, the hormones are effective at low concentrations. This would rule out compounds such as sucrose which is produced within the leaves by photosynthesis, is transported throughout the plant in the phloem, and has a profound effect on plant development, but is found at high concentrations. Sucrose has been considered by some to be a plant growth hormone. However, in this chapter the use of the term is limited to compounds which have traditionally been classed as plant growth hormones. These have the following characteristics which can be added to the definition above.

In general :

- Plant growth hormones are small, relatively simple compounds.
- Specific receptors exist which bind these compounds.
- Often the presence of one plant growth hormone will affect the synthesis or action of other plant growth hormones.

The concentration of a plant growth hormone at a particular site will depend upon many different factors including the rate of synthesis, degradation and transport to and from the target cell. In addition, plant growth hormones are often chemically modified, which may inactivate them, although – as this process is often reversible – it can also increase the effective concentration of a plant growth hormone in a cell. Finally, as the activity of plant hormones is thought to require binding to specific receptors, transport in and out of subcellular compartments also controls the concentration perceived by the cell. As all of these processes have the potential to be regulated by the environment, it can be seen that plant growth hormones act as a means of integrating environmental signals and distributing them around the plant.

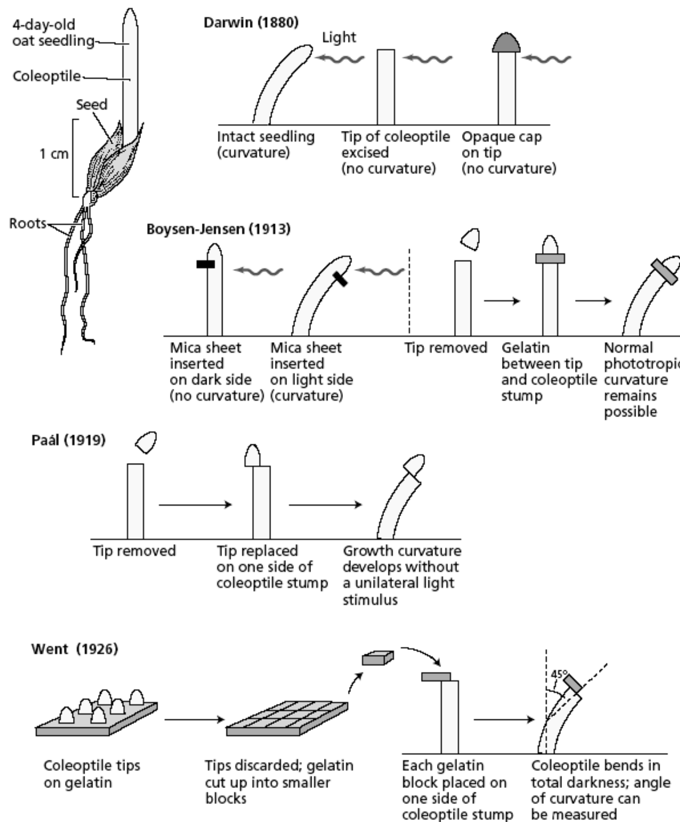
Classically there are five major groups of plant growth hormones. These are the auxins, gibberellins, cytokinins, abscisins and ethylene However, many more compounds exist in plants which have all of the characteristics of the more established plant growth hormones, and their role in regulating plant development is becoming clearer. These compounds include the jasmonates, salicylates, brassinosteroids and peptide hormones. Recently, small RNA molecules have been identified which regulate gene expression in different parts of the plants; these too might be considered to fulfil many of the criteria of being a plant growth hormone.

THE EMERGENCE OF THE AUXIN CONCEPT

During the latter part of the nineteenth century, Charles Darwin and his son Francis studied plant growth phenomena involving tropisms. One of their interests was the bending of plants toward light. This phenomenon, which is caused by differential growth, is called **phototropism**. In some experiments the Darwins used seedlings of canary grass (*Phalaris canariensis*), in which, as in many other grasses, the youngest leaves are sheathed in a protective organ called the **coleoptile** (Figure).

Coleoptiles are very sensitive to light, especially to blue light (see Chapter 18). If illuminated on one side with a short pulse of dim blue light, they will bend (grow) toward the source of the light pulse within an hour. The Darwins found that the tip of the coleoptile perceived the light, for if they covered the tip with foil, the coleoptile would not bend. But the region of the coleoptile that is responsible for the bending toward the light, called the **growth zone**, is several millimeters below the tip.

Thus they concluded that some sort of signal is produced in the tip, travels to the growth zone, and causes the shaded side to grow faster than the illuminated side. The results of their experiments were published in 1881 in a remarkable book entitled *The Power of Movement in Plants*. There followed a long period of experimentation by many investigators on the nature of the growth stimulus in coleoptiles. This research culminated in the demonstration in 1926 by Frits Went of the presence of a growth-promoting chemical in the tip of oat (*Avena sativa*) coleoptiles. It was known that if the tip of a coleoptile was removed, coleoptile growth ceased. Previous workers had attempted to isolate and identify the growth-promoting chemical by grinding up coleoptile tips and testing the activity of the extracts. This approach failed because grinding up the tissue released into the extract inhibitory substances that normally were compartmentalized in the cell. Went's major breakthrough was to avoid grinding by allowing the material to diffuse out of excised coleoptiles tips directly into gelatin blocks. If placed asymmetrically on top of a decapitated coleoptile, these blocks could be tested for their ability to cause bending in the absence of a unilateral light source (see Figure). Because the substance promoted the elongation of the coleoptile sections, it was eventually named **auxin** from the Greek *auxein*, meaning "to increase" or "to grow."

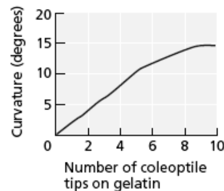


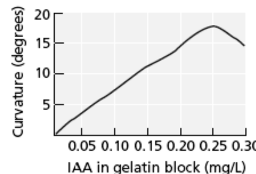
From experiments on coleoptile phototropism, Darwin concluded in 1880 that a growth stimulus is produced in the coleoptile tip and is transmitted to the growth zone.

In 1913, P. Boysen-Jensen discovered that the growth stimulus passes through gelatin but not through water-impermeable barriers such as mica.

In 1919, A. Paál provided evidence that the growth-promoting stimulus produced in the tip was chemical in nature.

In 1926, F. W. Went showed that the active growth-promoting substance can diffuse into a gelatin block. He also devised a coleoptile-bending assay for quantitative auxin analysis.





BIOSYNTHESIS AND METABOLISM OF AUXIN

Went's studies with agar blocks demonstrated unequivocally that the growth-promoting "influence" diffusing from the coleoptile tip was a chemical substance. The fact that it was produced at one location and transported in minute amounts to its site of action qualified it as an authentic plant hormone.

The Principal Auxin in Higher Plants Is Indole-3-Acetic Acid

In the mid-1930s it was determined that auxin is **indole-3-acetic acid** (**IAA**). Several other auxins in higher plants were discovered later (Figure), but IAA is by far the most abundant and physiologically relevant. Because the structure of IAA is relatively simple, academic and industrial laboratories were quickly able to synthesize a wide array of molecules with auxin activity. Some of these are used as herbicides in horticulture and agriculture.

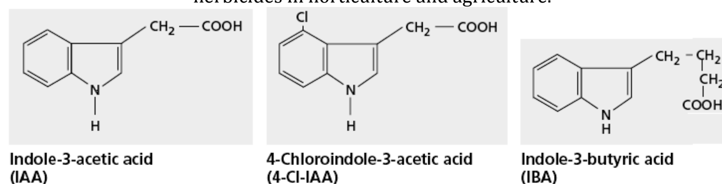


FIGURE Structure of three natural auxins. Indole-3-acetic acid (IAA) occurs in all plants, but other related compounds in plants have auxin activity. Peas, for example, contain 4-chloroindole-3-acetic acid. Mustards and corn contain indole-3-butyric acid (IBA).

An early definition of auxins included all natural and synthetic chemical substances that stimulate elongation in coleoptiles and stem sections. However, auxins affect many developmental processes besides cell elongation. Thus auxins can be defined as compounds with biological activities similar to those of IAA, including the ability to promote cell elongation in coleoptile and stem sections, cell division in callus cultures in the presence of cytokinins, formation of adventitious roots on detached leaves and stems, and other developmental phenomena associated with IAA action.

Although they are chemically diverse, a common feature of all active auxins is a molecular distance of about 0.5 nm between a fractional positive charge on the aromatic ring and a negatively charged carboxyl group.

Auxins in Biological Samples Can Be Quantified

Depending on the information that a researcher needs, the amounts and/or identity of auxins in biological samples can be determined by bioassay, mass spectrometry, or enzyme-linked immunosorbent assay, which is abbreviated as ELISA.

A **bioassay** is a measurement of the effect of a known or suspected biologically active substance on living material. In his pioneering work more than 60 years ago, Went used *Avena sativa* (oat) coleoptiles in a technique called the ***Avena* coleoptile curvature test**. The coleoptiles curved because the increase in auxin on one side stimulated cell elongation, and the decrease in auxin on the other side (due to the absence of the coleoptile tip) caused a decrease in the growth rate—a phenomenon called **differential growth**.

Went found that he could estimate the amount of auxin in a sample by measuring the resulting coleoptile curvature. Auxin bioassays are still used today to detect the presence of auxin activity in a sample. The *Avena* coleoptile curvature assay is a sensitive measure of auxin activity (it is effective for IAA concentrations of about 0.02 to 0.2 mg L–1). Another bioassay measures auxin-induced changes in the straight growth of *Avena* coleoptiles floating in solution. Both of these bioassays can establish the presence of an auxin in a sample, but they cannot be used for precise quantification or identification of the specific compound.

Mass spectrometry is the method of choice when information about both the chemical structure and the amount of IAA is needed. This method is used in conjunction with separation protocols involving gas chromatography. It allows the precise quantification and identification of auxins, and can detect as little as 10–12 g (1 picogram, or pg) of IAA, which is well within the range of auxin found in a single pea stem section or a corn kernel. These sophisticated techniques have enabled researchers to accurately analyze auxin precursors, auxin turnover, and auxin distribution within the plant.

IAA Is Synthesized in Meristems,Young Leaves, and Developing Fruits and Seeds

IAA biosynthesis is associated with rapidly dividing and rapidly growing tissues, especially in shoots. Although virtually all plant tissues appear to be capable of producing low levels of IAA, shoot apical meristems, young leaves, and developing fruits and seeds are the primary sites of IAA synthesis.

In very young leaf primordia of *Arabidopsis*, auxin is synthesized at the tip. During leaf development there is a gradual shift in the site of auxin production basipetally along the margins, and later, in the central region of the lamina. The basipetal shift in auxin production correlates closely with, and is probably causally related to, the basipetal maturation sequence of leaf development and vascular differentiation.

By fusing the *GUS* (β-glucuronidase) reporter gene to a promoter containing an auxin response element, and transforming *Arabidopsis* leaves with this construct in a Ti plasmid using *Agrobacterium*, it is possible to visualize the distribution of free auxin in young, developing leaves. Wherever free auxin is produced, *GUS* expression occurs—and can be detected histochemically. By use of this technique, it has recently been demonstrated that auxin is produced by a cluster of cells located at sites where hydathodes will develop.

Hydathodes are glandlike modifications of the ground and vascular tissues, typically at the margins of leaves, that allow the release of liquid water (guttation fluid) through pores in the epidermis in the presence of root pressure.

Multiple Pathways Exist for the Biosynthesis of IAA

IAA is structurally related to the amino acid tryptophan, and early studies on auxin biosynthesis focused on tryptophan as the probable precursor. However, the incorporation of exogenous labeled tryptophan (e.g., [^{3}H]tryptophan) into IAA by plant tissues has proved difficult to demonstrate. Nevertheless, an enormous body of evidence has now accumulated showing that plants convert tryptophan to IAA by several pathways, which are described in the paragraphs that follow.

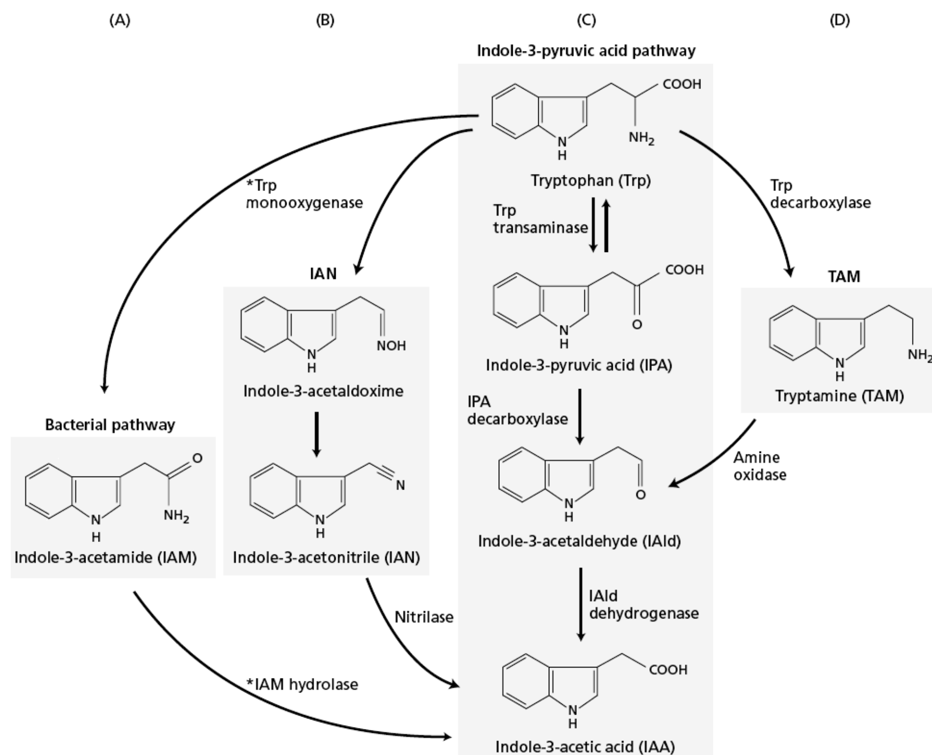


FIGURE Tryptophan-dependent pathways of IAA biosynthesis in plants and bacteria. The enzymes that are present only in bacteria are marked with an asterisk.

The IPA pathway. The **indole-3-pyruvic acid** (**IPA**) pathway (see Figure C), is probably the most common of the tryptophan-dependent pathways. It involves a deamination reaction to form IPA, followed by a decarboxylation reaction to form indole-3-acetaldehyde (IAld). Indole-3- acetaldehyde is then oxidized to IAA by a specific dehydrogenase.

The TAM pathway. The **tryptamine** (**TAM**) **pathway** (see Figure D) is similar to the IPA pathway, except that the order of the deamination and decarboxylation reactions is reversed, and different enzymes are involved. Species that do not utilize the IPApathway possess the TAM pathway. In at least one case (tomato), there is evidence for both the IPAand the TAM pathways.

The IAN pathway. In the **indole-3-acetonitrile** (**IAN**) pathway (see Figure B), tryptophan is first converted to indole-3-acetaldoxime and then to indole-3-acetonitrile. The enzyme that converts IAN to IAA is called *nitrilase*.

The IAN pathway may be important in only three plant families: the Brassicaceae (mustard family), Poaceae (grass family), and Musaceae (banana family). Nevertheless, nitrilase-like genes or activities have recently been identified in the Cucurbitaceae (squash family), Solanaceae (tobacco family), Fabaceae (legumes), and Rosaceae (rose family). Four genes (*NIT1* through *NIT4*) that encode nitrilase enzymes have now been cloned from *Arabidopsis*.

When *NIT2* was expressed in transgenic tobacco, the resultant plants acquired the ability to respond to IAN as an auxin by hydrolyzing it to IAA.

Another tryptophan-dependent biosynthetic pathway— one that uses **indole-3-acetamide** (**IAM**) as an intermediate (see Figure A)—is used by various pathogenic bacteria, such as *Pseudomonas savastanoi* and *Agrobacterium tumefaciens*. This pathway involves the two enzymes tryptophan monooxygenase and IAM hydrolase. The auxins produced by these bacteria often elicit morphological changes in their plant hosts.

In addition to the tryptophan-dependent pathways, recent genetic studies have provided evidence that plants can synthesize IAA via one or more tryptophan-independent pathways. The existence of multiple pathways for IAA biosynthesis makes it nearly impossible for plants to run out of auxin and is probably a reflection of the essential role of this hormone in plant development.

IAA Is Also Synthesized from Indole or from Indole-3-Glycerol Phosphate

Although a tryptophan-independent pathway of IAA biosynthesis had long been suspected because of the low levels of conversion of radiolabeled tryptophan to IAA, not until genetic approaches were available could the existence of such pathways be confirmed and defined. Perhaps the most striking of these studies in maize involves the *orange pericarp* (*orp*) mutant, in which both subunits of the enzyme tryptophan synthase are inactive (Figure). The *orp* mutant is a true tryptophan auxotroph, requiring exogenous tryptophan to survive. However, neither the *orp* seedlings nor the wild-type seedlings can convert tryptophan to IAA, even when the mutant seedlings are given enough tryptophan to reverse the lethal effects of the mutation.

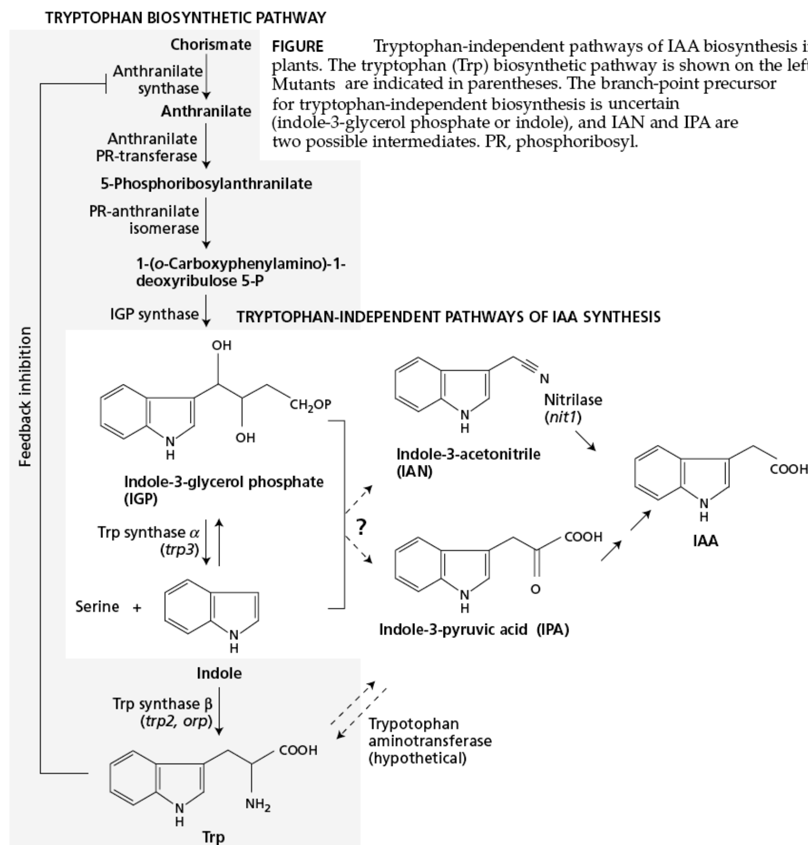


FIGURE Tryptophan-independent pathways of IAA biosynthesis in plants. The tryptophan (Trp) biosynthetic pathway is shown on the left. Mutants are indicated in parentheses. The branch-point precursor for tryptophan-independent biosynthesis is uncertain (indole-3-glycerol phosphate or indole), and IAN and IPA are two possible intermediates. PR, phosphoribosyl.

Despite the block in tryptophan biosynthesis, the *orp* mutant contains amounts of IAA 50-fold higher than those of a wild-type plant. Signficantly, when *orp* seedlings were fed [^{15}N]anthranilate (see Figure), the label subsequently appeared in IAA, but not in tryptophan. These results provided the best experimental evidence for a tryptophan-independent pathway of IAA biosynthesis.

Further studies established that the branch point for IAA biosynthesis is either indole or its precursor, indole-3-glycerol phosphate (see Figure). IAN and IPA are possible intermediates, but the immediate precursor of IAA in the tryptophan-independent pathway has not yet been identified.

The discovery of the tryptophan-independent pathway has drastically altered our view of IAA biosynthesis, but the relative importance of the two pathways (tryptophan dependent versus tryptophan-independent) is poorly understood. In several plants it has been found that the type of IAA biosynthesis pathway varies between different tissues, and between different times of development. For example, during embryogenesis in carrot, the tryptophan dependent pathway is important very early in development, whereas the tryptophan-independent pathway takes over soon after the root–shoot axis is established.

Most IAA in the Plant Is in a Covalently Bound Form

Although free IAA is the biologically active form of the hormone, the vast majority of auxin in plants is found in a covalently bound state. These conjugated, or "bound," auxins have been identified in all higher plants and are considered hormonally inactive.

IAA has been found to be conjugated to both high- and low-molecular-weight compounds.

- Low-molecular-weight conjugated auxins include esters of IAA with glucose or *myo*-inositol and amide conjugates such as IAA-*N*-aspartate.
- High-molecular-weight IAA conjugates include IAA glucan (7–50 glucose units per IAA) and IAA-glycoproteins found in cereal seeds.

The compound to which IAA is conjugated and the extent of the conjugation depend on the specific conjugating enzymes. The best-studied reaction is the conjugation of IAA to glucose in *Zea mays*.

The highest concentrations of free auxin in the living plant are in the apical meristems of shoots and in young leaves because these are the primary sites of auxin synthesis. However, auxins are widely distributed in the plant.

Metabolism of conjugated auxin may be a major contributing factor in the regulation of the levels of free auxin. For example, during the germination of seeds of *Zea mays*, IAA-*myo*-inositol is translocated from the endosperm to the coleoptile via the phloem. At least a portion of the free IAA produced in coleoptile tips of *Zea mays* is believed to be derived from the hydrolysis of IAA-*myo*-inositol.

In addition, environmental stimuli such as light and gravity have been shown to influence both the rate of auxin conjugation (removal of free auxin) and the rate of release of

free auxin (hydrolysis of conjugated auxin). The formation of conjugated auxins may serve other functions as well, including storage and protection against oxidative degradation.

IAA Is Degraded by Multiple Pathways

Like IAA biosynthesis, the enzymatic breakdown (oxidation) of IAA may involve more than one pathway. For some time it has been thought that peroxidative enzymes are chiefly responsible for IAA oxidation, primarily because these enzymes are ubiquitous in higher plants and their ability to degrade IAA can be demonstrated in vitro. However, the physiological significance of the peroxidase pathway is unclear. For example, no change in the IAA levels of transgenic plants was observed with either a tenfold increase in peroxidase expression or a tenfold repression of peroxidase activity.

On the basis of isotopic labeling and metabolite identification, two other oxidative pathways are more likely to be involved in the controlled degradation of IAA. The end product of this pathway is oxindole-3-acetic acid (OxIAA), a naturally occurring compound in the endosperm and shoot tissues of *Zea mays*. In one pathway, IAA is oxidized without decarboxylation to OxIAA.

In another pathway, the IAA-aspartate conjugate is oxidized first to the intermediate dioxindole-3-acetylaspartate, and then to OxIAA.

In vitro, IAA can be oxidized nonenzymatically when exposed to high-intensity light, and its photodestruction in vitro can be promoted by plant pigments such as riboflavin. Although the products of auxin photooxidation have been isolated from plants, the role, if any, of the photooxidation pathway in vivo is presumed to be minor.

Two Subcellular Pools of IAA Exist: The Cytosol and the Chloroplasts

The distribution of IAA in the cell appears to be regulated largely by pH. Because IAA− does not cross membranes unaided, whereas IAAH readily diffuses across membranes, auxin tends to accumulate in the more alkaline compartments of the cell.

The distribution of IAA and its metabolites has been studied in tobacco cells. About one-third of the IAA is found in the chloroplast, and the remainder is located in the cytosol. IAA conjugates are located exclusively in the cytosol. IAA in the cytosol is metabolized either by conjugation or by nondecarboxylative catabolism.

The IAA in the chloroplast is protected from these processes, but it is regulated by the amount of IAA in the cytosol, with which it is in equilibrium.

AUXIN TRANSPORT

The main axes of shoots and roots, along with their branches, exhibit apex–base structural polarity, and this structural polarity has its origin in the polarity of auxin transport. Soon after Went developed the coleoptile curvature test for auxin, it was discovered that IAA moves mainly from the apical to the basal end (*basipetally*) in excised oat coleoptile sections. This type of unidirectional transport is termed **polar transport**. Auxin is the only plant growth hormone known to be transported polarly.

Polar Transport Requires Energy and Is Gravity Independent

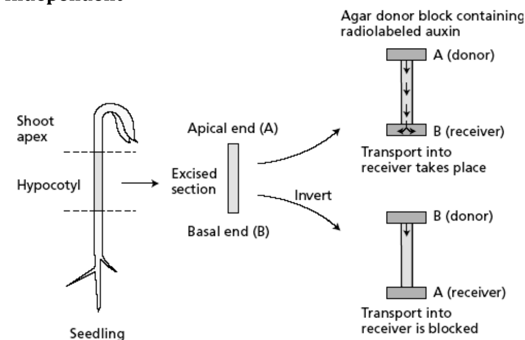


FIGURE The standard method for measuring polar auxin transport. The polarity of transport is independent of orientation with respect to gravity.

To study polar transport, researchers have employed the *donor–receiver agar block method* (Figure): An agar block containing radioisotope-labeled auxin (donor block) is placed on one end of a tissue segment, and a receiver block is placed on the other end. The movement of auxin through the tissue into the receiver block can be determined over time by measurement of the radioactivity in the receiver block.

From a multitude of such studies, the general properties of polar IAA transport have emerged. Tissues differ in the degree of polarity of IAA transport. In coleoptiles, vegetative stems, and leaf petioles, basipetal transport predominates. Polar transport is not affected by the orientation of the tissue (at least over short periods of time), so it is independent of gravity.

When stem cuttings (in this case bamboo) are placed in a moist chamber, adventitious roots always form at the basal end of the cuttings, even when the cuttings are inverted. Because root differentiation is stimulated by an increase in auxin concentration, auxin must be transported basipetally in the stem even when the cutting is oriented upside down.

Polar transport proceeds in a cell-to-cell fashion, rather than via the symplast. That is, auxin exits the cell through the plasma membrane, diffuses across the compound middle lamella, and enters the cell below through its plasma membrane. The loss of auxin from cells is termed *auxin efflux*; the entry of auxin into cells is called *auxin uptake* or *influx*. The overall process requires metabolic energy, as evidenced by the sensitivity of polar transport to O_2 deprivation and metabolic inhibitors.

The velocity of polar auxin transport is 5 to 20 cm h^{-1}— faster than the rate of diffusion, but slower than phloem translocation rates.

Polar transport is also specific for active auxins, both natural and synthetic. Neither inactive auxin analogs nor auxin metabolites are transported polarly, suggesting that polar transport involves specific protein carriers on the plasma membrane that can recognize the hormone and its active analogs.

The major site of basipetal polar auxin transport in stems and leaves is the vascular parenchyma tissue. Coleoptiles appear to be the exception in that basipetal polar transport

occurs mainly in the nonvascular tissues. Acropetal polar transport in the root is specifically associated with the xylem parenchyma of the stele.

However, most of the auxin that reaches the root tip is translocated via the phloem.

A small amount of basipetal auxin transport from the root tip has also been demonstrated. In maize roots, for example, radiolabeled IAA applied to the root tip is transported basipetally about 2 to 8 mm. Basipetal auxin transport in the root occurs in the epidermal and cortical tissues, and as we shall see, it plays a central role in gravitropism.

A Chemiosmotic Model Has Been Proposed to Explain Polar Transport

The discovery of the chemiosmotic mechanism of solute transport in the late 1960s led to the application of this model to polar auxin transport. According to the now generally accepted **chemiosmotic model** for polar auxin transport, auxin uptake is driven by the proton motive force (ΔE + ΔpH) across the plasma membrane, while auxin efflux is driven by the membrane potential, ΔE.

Acrucial feature of the polar transport model is that the auxin efflux carriers are localized at the basal ends of the conducting cells. The evidence for each step in this model is considered separately in the discussion that follows.

Auxin influx. The first step in polar transport is auxin influx. According to the model, auxin can enter plant cells from any direction by either of two mechanisms:

1. Passive diffusion of the protonated (IAAH) form across the phospholipid bilayer
2. Secondary active transport of the dissociated (IAA^-) form via a $2H^+$–IAA– symporter. The dual pathway of auxin uptake arises because the passive permeability of the membrane to auxin depends strongly on the apoplastic pH.

The undissociated form of indole-3-acetic acid, in which the carboxyl group is protonated, is lipophilic and readily diffuses across lipid bilayer membranes. In contrast, the dissociated form of auxin is negatively charged and therefore does not cross membranes unaided. Because the plasma membrane H^+-ATPase normally maintains the cell wall solution at about pH 5, about half of the auxin (pKa = 4.75) in the apoplast will be in the undissociated form and will diffuse passively across the plasma membrane down a concentration gradient. Experimental support for pH-dependent, passive auxin uptake was first provided by the demonstration that IAA uptake by plant cells increases as the extracellular pH is lowered from a neutral to a more acidic value.

A carrier-mediated, secondary active uptake mechanism was shown to be saturable and specific for active auxins. In experiments in which the ΔpH and ΔE values of isolated membrane vesicles from zucchini (*Cucurbita pepo*) hypocotyls were manipulated artificially, the uptake of radiolabeled auxin was shown to be stimulated in the presence of a pH gradient, as in passive uptake, but also when the inside of the vesicle was negatively charged relative to the outside.

These and other experiments suggested that an H^+–IAA– symporter cotransports two protons along with the auxin anion. This secondary active transport of auxin allows for greater auxin accumulation than simple diffusion does because it is driven across the membrane by the proton motive force.

A permease-type auxin uptake carrier, AUX1, related to bacterial amino acid carriers, has been identified in *Arabidopsis* roots. The roots of *aux1* mutants are agravitropic, suggesting that auxin influx is a limiting factor for gravitropism in roots. As predicted by the chemiosmotic model, AUX1 appears to be uniformly distributed around cells in the polar transport pathway. Thus in general, the polarity of auxin transport is governed by the efflux step rather than the influx step.

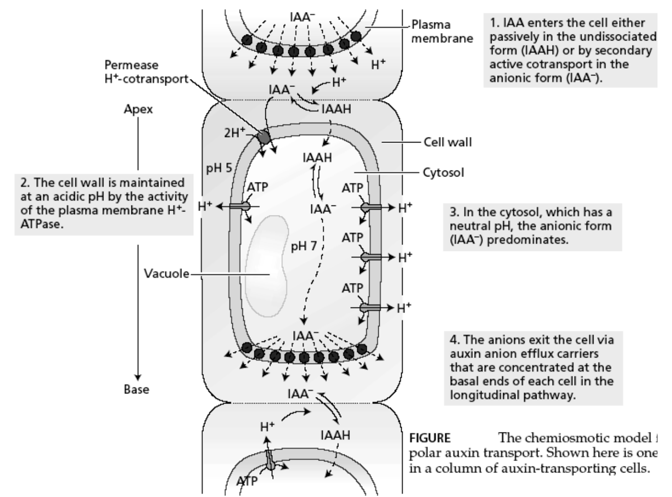


FIGURE The chemiosmotic model f polar auxin transport. Shown here is one in a column of auxin-transporting cells.

Auxin efflux. Once IAA enters the cytosol, which has a pH of approximately 7.2, nearly all of it will dissociate to the anionic form. Because the membrane is less permeable to IAA^- than to IAAH, IAA^- will tend to accumulate in the cytosol. However, much of the auxin that enters the cell escapes via an *auxin anion efflux carrier*.

According to the chemiosmotic model, transport of IAA^- out of the cell is driven by the inside negative membrane potential.

As noted earlier, the central feature of the chemiosmotic model for polar transport is that IAA– efflux takes place preferentially at the basal end of each cell. The repetition of auxin uptake at the apical end of the cell and preferential release from the base of each cell in the pathway gives rise to the total polar transport effect. A family of putative auxin efflux carriers known as **PIN proteins** (named after the pin-shaped inflorescences formed by the *pin1* mutant of *Arabidopsis*) are localized precisely as the model would predict—that is, at the basal ends of the conducting cells.

Inhibitors of Auxin Transport Block Auxin Efflux

Several compounds have been synthesized that can act as **auxin transport inhibitors** (**ATIs**), including **NPA (1-*N*naphthylphthalamic acid) and TIBA (2,3,5-triiodobenzoic acid).** These inhibitors block polar transport by preventing auxin efflux. We can demonstrate this phenomenon by incorporating NPA or TIBA into either the donor or the receiver block in an auxin transport experiment.

Both compounds inhibit auxin efflux into the receiver block, but they do not affect auxin uptake from the donor block. Some ATIs, such as TIBA, that have weak auxin activity and are transported polarly, may inhibit polar transport in part by competing with auxin for its binding site on the efflux carrier. Others, such as NPA, are not transported polarly and are believed to interfere with auxin transport by binding to proteins associated in a complex with the efflux carrier. Such NPA-binding proteins are also found at the basal ends of the conducting cells, consistent with the localization of PIN proteins.

Recently another class of ATIs has been identified that inhibits the AUX1 uptake carrier. For example, 1-naphthoxyacetic acid (1-NOA) blocks auxin uptake into cells, and when applied to *Arabidopsis* plants it causes root agravitropism similar to that of the *aux1* mutant. Like the *aux1* mutation, neither 1- NOA nor any of the other AUX1-specific inhibitors block polar auxin transport.

PIN Proteins Are Rapidly Cycled to and from the Plasma Membrane

The basal localization of the auxin efflux carriers involves targeted vesicle secretion to the basal ends of the conducting cells. Recently it has been demonstrated that PIN proteins, although stable, do not remain on the plasma membrane permanently, but are rapidly cycled to an unidentified endosomal compartment via endocytotic vesicles, and then recycled back to the plasma membrane.

Prior to treatment, the PIN1 protein is localized at the basal ends (top) of root cortical parenchyma cells. Treatment of *Arabidopsis* seedlings with brefeldin A(BFA), which causes Golgi vesicles and other endosomal compartments to aggregate near the nucleus, causes PIN to accumulate in these abnormal intracellular compartments. When the BFA is washed out with buffer, the normal localization on the plasma membrane at the base of the cell is restored.

But when cytochalasin D, an inhibitor of actin polymerization, is included in the buffer washout solution, normal relocalization of PIN to the plasma membrane is prevented. These results indicate that PIN is rapidly cycled between the plasma membrane at the base of the cell and an unidentified endosomal compartment by an actin-dependent mechanism.

Although they bind different targets, both TIBA and NPA interfere with vesicle traffic to and from the plasma membrane.

The best way to demonstrate this phenomenon is to include TIBA in the washout solution after BFA treatment. Under these conditions, TIBA prevents the normal relocalization of PIN on the plasma membrane following the washout treatment.

The effects of TIBA and NPA on cycling are not specific for PIN proteins, and it has been proposed that ATIs may actually represent general inhibitors of membrane cycling. On the other hand, neither TIBA nor NPA alone causes PIN delocalization, even though they block auxin efflux. Therefore, TIBA and NPA must also be able to directly inhibit the transport activity of PIN complexes on the plasma membrane—by binding either to PIN (as TIBA does) or to one or more regulatory proteins (as NPA does).

A simplified model of the effects of TIBA and NPA on PIN cycling and auxin efflux is shown in Figure.

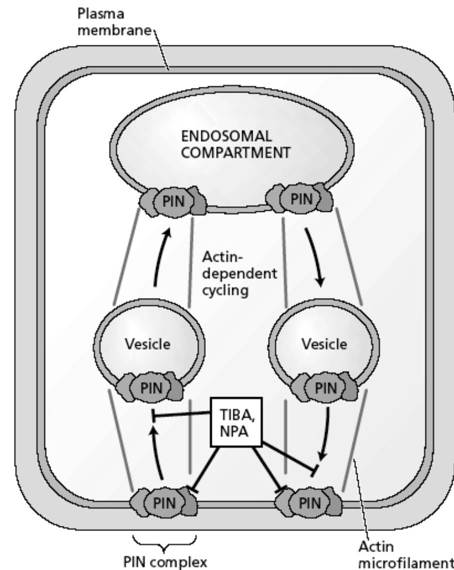


FIGURE Actin-dependent PIN cycling between the plasma membrane and an endosomal compartment. Auxin transport inhibitors TIBA and NPA both interfere with relocalization of PIN1 proteins to basal plasma membranes after BFA washout (see Figure 19.17). This suggests that both of these auxin transport inhibitors interfere with PIN1 cycling.

Flavonoids Serve as Endogenous ATIs

There is mounting evidence that flavonoids can function as endogenous regulators of polar auxin transport. Indeed, naturally occurring aglycone flavonoid compounds (flavonoids without attached sugars) are able to compete with NPA for its binding site on membranes and are typically localized on the plasma membrane at the basal ends of cells where the efflux carrier is concentrated.

Auxin Is Also Transported Nonpolarly in the Phloem

Most of the IAA that is synthesized in mature leaves appears to be transported to the rest of the plant nonpolarly via the phloem. Auxin, along with other components of phloem sap, can move from these leaves up or down the plant at velocities much higher than those of polar transport. Auxin translocation in the phloem is largely passive, not requiring energy directly.

Although the overall importance of the phloem pathway versus the polar transport system for the long-distance movement of IAA in plants is still unresolved, the evidence suggests that long-distance auxin transport in the phloem is important for controlling such processes as cambial cell divisions, callose accumulation or removal from sieve tube elements, and branch root formation. Indeed, the phloem appears to represent the principal pathway for long-distance auxin translocation to the root.

Polar transport and phloem transport are not independent of each other. Recent studies with radiolabeled IAA suggest that in pea, auxin can be transferred from the nonpolar phloem pathway to the polar transport pathway. This transfer takes place mainly in the immature tissues of the shoot apex.

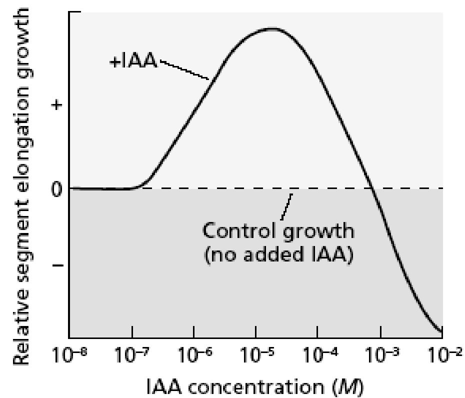
PHYSIOLOGICAL EFFECTS OF AUXIN:

Auxins Promote Growth in Stems and Coleoptiles, While Inhibiting Growth in Roots

As we have seen, auxin is synthesized in the shoot apex and transported basipetally to the tissues below. The steady supply of auxin arriving at the subapical region of the stem or coleoptile is required for the continued elongation of these cells. Because the level of endogenous auxin in the elongation region of a normal healthy plant is nearly optimal for growth, spraying the plant with exogenous auxin causes only a modest and short-lived stimulation in growth, and may even be inhibitory in the case of darkgrown seedlings, which are more sensitive to supraoptimal auxin concentrations than light-grown plants are.

However, when the endogenous source of auxin is removed by excision of sections containing the elongation zones, the growth rate rapidly decreases to a low basal rate. Such excised sections will often respond dramatically to exogenous auxin by rapidly increasing their growth rate back to the level in the intact plant.

In long-term experiments, treatment of excised sections of coleoptiles or dicot stems with auxin stimulates the rate of elongation of the section for up to 20 hours. The optimal auxin concentration for elongation growth is typically 10^{-6} to 10^{-5} *M*(Figure).



The inhibition beyond the optimal concentration is generally attributed to auxin-induced ethylene biosynthesis. Auxin control of root elongation growth has been more difficult to demonstrate, perhaps because auxin induces the production of ethylene, a root growth inhibitor. However, even if ethylene biosynthesis is specifically blocked, low concentrations (10^{-10} to 10^{-9} *M*) of auxin promote the growth of intact roots, whereas higher concentrations (10^{-6} *M*) inhibit growth. Thus, roots may require a minimum concentration of auxin to grow, but root growth is strongly inhibited by auxin concentrations that promote elongation in stems and coleoptiles.

The Outer Tissues of Dicot Stems Are the Targets of Auxin Action

Dicot stems are composed of many types of tissues and cells, only some of which may limit the growth rate. This point is illustrated by a simple experiment. When stem sections from growing regions of an etiolated dicot stem, such as pea, are split lengthwise and incubated in buffer, the two halves bend outward.

This result indicates that, in the absence of auxin the central tissues, including the pith, vascular tissues, and inner cortex, elongate at a faster rate than the outer tissues, consisting of the outer cortex and epidermis. Thus the outer tissues must be limiting the extension rate of the stem in the absence of auxin. However, when the split sections are incubated in buffer plus auxin, the two halves now curve inward, demonstrating that the outer tissues of dicot stems are the primary targets of auxin action during cell elongation.

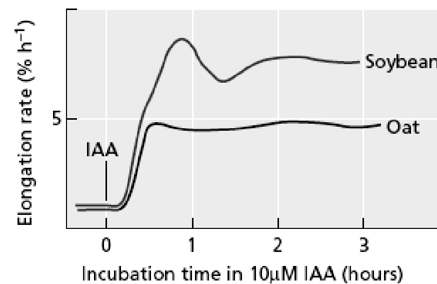
The observation that the outer cell layers are the targets of auxin seems to conflict with the localization of polar transport in the parenchyma cells of the vascular bundles.

However, auxin can move laterally from the vascular tissues of dicot stems to the outer tissues of the elongation zone. In coleoptiles, on the other hand, all of the nonvascular tissues (epidermis plus mesophyll) are capable of transporting auxin, as well as responding to it.

The Minimum Lag Time for Auxin-Induced Growth Is Ten Minutes

When a stem or coleoptile section is excised and inserted into a sensitive growth-measuring device, the growth response to auxin can be monitored at very high resolution. Without auxin in the medium, the growth rate declines rapidly. Addition of auxin markedly stimulates the growth rate after a lag period of only 10 to 12 minutes.

Both *Avena* (oat) coleoptiles and *Glycine max* (soybean) hypocotyls (dicot stem) reach a maximum growth rate after 30 to 60 minutes of auxin treatment (Figure).



This maximum represents a five- to tenfold increase over the basal rate. Oat coleoptile sections can maintain this maximum rate for up to 18 hours in the presence of osmotically active solutes such as sucrose or KCl.

As might be expected, the stimulation of growth by auxin requires energy, and metabolic inhibitors inhibit the response within minutes. Auxin-induced growth is also sensitive to inhibitors of protein synthesis such as cycloheximide, suggesting that proteins with high turnover rates are involved. Inhibitors of RNA synthesis also inhibit auxininduced growth, after a slightly longer delay.

Although the length of the lag time for auxin-stimulated growth can be increased by lowering of the temperature or by the use of suboptimal auxin concentrations, the lag time cannot be shortened by raising of the temperature, by the use of supraoptimal auxin concentrations, or by abrasion of the waxy cuticle to allow auxin to penetrate the tissue more rapidly. Thus the minimum lag time of 10 minutes is not determined by the time required for auxin to reach its site of action. Rather, the lag time reflects the time needed for the biochemical machinery of the cell to bring about the increase in the growth rate.

Auxin Rapidly Increases the Extensibility of the Cell Wall

How does auxin cause a five- to tenfold increase in the growth rate in only 10 minutes? To understand the mechanism, we must first review the process of cell enlargement in plants. Plant cells expand in three steps:
1. Osmotic uptake of water across the plasma membrane is driven by the gradient in water potential (ΔYw).
2. Turgor pressure builds up because of the rigidity of the cell wall.
3. Biochemical wall loosening occurs, allowing the cell to expand in response to turgor pressure.

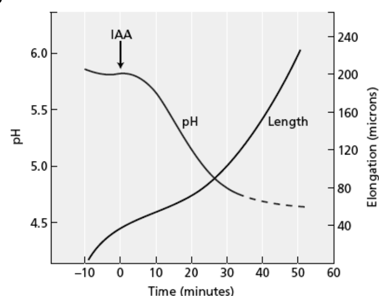
The effects of these parameters on the growth rate are encapsulated in the growth rate equation:
$GR = m\ (Y\text{p} - Y)$
where GR is the growth rate, Yp is the turgor pressure, Y is the yield threshold, and m is the coefficient (*wall extensibility*) that relates the growth rate to the difference between Yp and Y.

In principle, auxin could increase the growth rate by increasing m, increasing Yp, or decreasing Y. Although extensive experiments have shown that auxin does not increase turgor pressure when it stimulates growth, conflicting results have been obtained regarding auxin induced decreases in Y. However, there is general agreement that auxin causes an increase in the wall extensibility parameter, m.

Auxin-Induced Proton Extrusion Acidifies the Cell Wall and Increases Cell Extension

According to the widely accepted **acid growth hypothesis**, hydrogen ions act as the intermediate between auxin and cell wall loosening. The source of the hydrogen ions is the plasma membrane H^+-ATPase, whose activity is thought to increase in response to auxin. The acid growth hypothesis allows five main predictions:
1. Acid buffers alone should promote short-term growth, provided the cuticle has been abraded to allow the protons access to the cell wall.
2. Auxin should increase the rate of proton extrusion (wall acidification), and the kinetics of proton extrusion should closely match those of auxin-induced growth.
3. Neutral buffers should inhibit auxin-induced growth.
4. Compounds (other than auxin) that promote proton extrusion should stimulate growth.
5. Cell walls should contain a "wall loosening factor" with an acidic pH optimum.

All five of these predictions have been confirmed. Acidic buffers cause a rapid and immediate increase in the growth rate, provided the cuticle has been abraded. Auxin stimulates proton extrusion into the cell wall after 10 to 15 minutes of lag time, consistent with the growth kinetics (Figure).



Auxin-induced growth has also been shown to be inhibited by neutral buffers, as long as the cuticle has been abraided. **Fusicoccin**, a fungal phytotoxin, stimulates both rapid proton extrusion and transient growth in stem and coleoptile sections. And finally, wall loosening proteins called **expansins** have been identified in the cell walls of a wide range of plant species. At acidic pH values, expansins loosen cell walls by weakening the hydrogen bonds between the polysaccharide components of the wall.

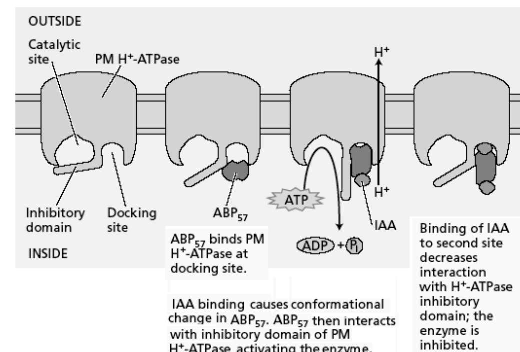
Auxin-Induced Proton Extrusion May Involve Both Activation and Synthesis

In theory, auxin could increase the rate of proton extrusion by two possible mechanisms:
1. Activation of preexisting plasma membrane H^+- ATPases
2. Synthesis of new H+-ATPases on the plasma membrane

H+-ATPase activation. When auxin was added directly to isolated plasma membrane vesicles from tobacco cells, a small stimulation (about 20%) of the ATP-driven proton-pumping activity was observed, suggesting that auxin directly activates the H^+-ATPase. A greater stimulation (about 40%) was observed if the living cells were treated with IAA just before the membranes were isolated, suggesting that a cellular factor is also required.

Although an auxin receptor has not yet been unequivocally identified, various auxin-binding proteins (ABPs) have been isolated and appear to be able to activate the plasma membrane H^+-ATPase in the presence of auxin.

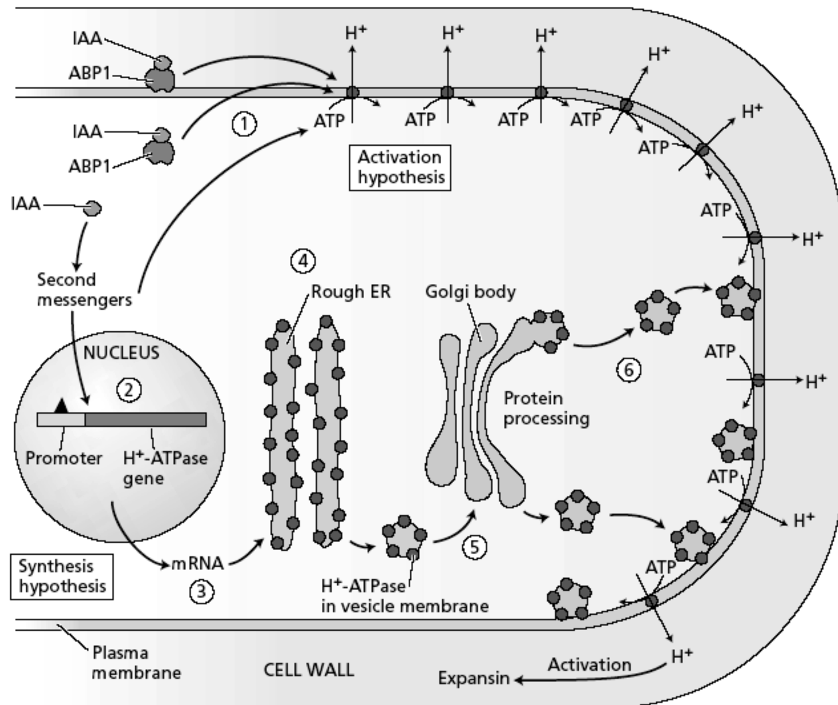
Recently an ABP from rice, ABP57, was shown to bind directly to plasma membrane H^+-ATPases and stimulate proton extrusion—but only in the presence of IAA. When IAA is absent, the activity of the H^+- ATPase is repressed by the C-terminal domain of the enzyme, which can block the catalytic site. ABP57 (with bound IAA) interacts with the H+-ATPase, activating the enzyme. A second auxin-binding site interferes with the action of the first, possibly explaining the bell-shaped curve of auxin action. This hypothetical model for the action of ABP57 is shown in Figure.



H+-ATPase synthesis. The ability of protein synthesis inhibitors, such as cycloheximide, to rapidly inhibit auxin induced proton extrusion and growth suggests that auxin might also stimulate proton pumping by increasing the synthesis of the H^+-ATPase. An increase in the amount of plasma membrane ATPase in corn coleoptiles was detected immunologically after only 5 minutes of auxin treatment, and a doubling of the H^+-ATPase was observed after 40 minutes of treatment. A threefold stimulation by auxin of an mRNA for the H^+-ATPase was demonstrated specifically in the nonvascular tissues of the coleoptiles.

Activation hypothesis: Auxin binds to an auxin-binding protein (ABP1) located either on the cell surface or in the cytosol. ABP1-IAA then interacts directly with plasma membrane H⁺-ATPase to stimulate proton pumping (step 1). Second messengers, such as calcium or intracellular pH, could also be involved.

Synthesis hypothesis: IAA-induced second messengers activate the expression of genes (step 2) that encode the plasma membrane H⁺-ATPase (step 3). The protein is synthesized on the rough endoplasmic reticulum (step 4) and targeted via the secretory pathway to the plasma membrane (steps 5 and 6). The increase in proton extrusion results from an increase in the number of proton pumps on the membrane.



In summary, the question of activation versus synthesis is still unresolved, and it is possible that auxin stimulates proton extrusion by both activation and stimulation of synthesis of the H^+-ATPase. Figure summarizes the proposed mechanisms of auxin-induced cell wall loosening via proton extrusion.

PHYSIOLOGICAL EFFECTS OF AUXIN: PHOTOTROPISM AND GRAVITROPISM

Three main guidance systems control the orientation of plant growth:
1. **Phototropism**, or growth with respect to light, is expressed in all shoots and some roots; it ensures that leaves will receive optimal sunlight for photosynthesis.
2. **Gravitropism**, growth in response to gravity, enables roots to grow downward into the soil and shoots to grow upward away from the soil, which is especially critical during the early stages of germination.
3. **Thigmotropism**, or growth with respect to touch, enables roots to grow around rocks and is responsible for the ability of the shoots of climbing plants to wrap around other structures for support.

Phototropism Is Mediated by the Lateral Redistribution of Auxin

As we saw earlier, Charles and Francis Darwin provided the first clue concerning the mechanism of phototropism by demonstrating that the sites of perception and differential growth (bending) are separate: Light is perceived at the tip, but bending occurs below the tip. The Darwins proposed that some "influence" that was transported from the tip to the growing region brought about the observed asymmetric growth response. This influence was later shown to be indole-3-acetic acid—auxin. When a shoot is growing vertically, auxin is transported polarly from the growing tip to the elongation zone. The polarity of auxin transport from tip to base is developmentally determined and is independent of orientation with respect to gravity. However, auxin can also be transported laterally, and this lateral movement of auxin lies at the heart of a model for tropisms originally proposed separately by the Russian plant physiologist, Nicolai Cholodny and Frits Went from the Netherlands in the 1920s.

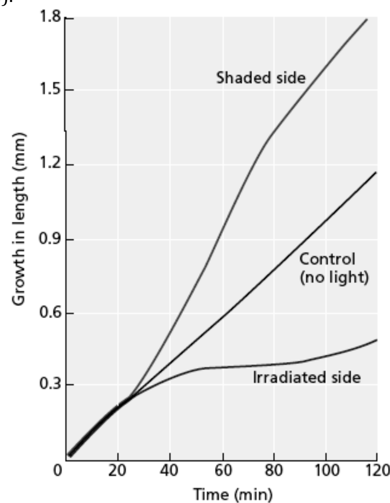
According to the Cholodny–Went model of phototropism, the tips of grass coleoptiles have three specialized functions:
1. The production of auxin
2. The perception of a unilateral light stimulus
3. The lateral transport of IAA in response to the phototropic stimulus

Thus, in response to a directional light stimulus, the auxin produced at the tip, instead of being transported basipetally, is transported laterally toward the shaded side. The precise sites of auxin production, light perception, and lateral transport have been difficult to define. In maize coleoptiles, auxin is produced in the upper 1 to 2 mm of the tip. The zones of photosensing and lateral transport extend farther, within the upper 5 mm of the tip. The response is also strongly dependent on the light fluence.

Two flavoproteins, *phototropins 1* and *2*, are the photoreceptors for the blue-light signaling pathway that induces phototropic bending in *Arabidopsis* hypocotyls and oat coleoptiles under both high- and low-fluence conditions.

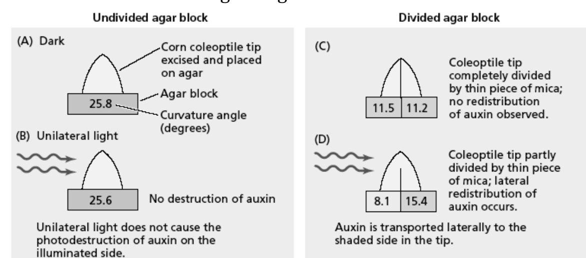
Phototropins are autophosphorylating protein kinases whose activity is stimulated by blue light. The action spectrum for **blue-light** activation of the kinase activity closely matches the action spectrum for phototropism, including the multiple peaks in the blue region. Phototropin 1 displays a lateral gradient in phosphorylation during exposure to low-fluence unilateral blue light.

According to the current hypothesis, the gradient in phototropin phosphorylation induces the movement of auxin to the shaded side of the coleoptile. Once the auxin reaches the shaded side of the tip, it is transported basipetally to the elongation zone, where it stimulates cell elongation. The acceleration of growth on the shaded side and the slowing of growth on the illuminated side (differential growth) give rise to the curvature toward light (Figure).



Direct tests of the Cholodny–Went model using the agar block/coleoptile curvature bioassay have supported the model's prediction that auxin in coleoptile tips is transported laterally in response to unilateral light. The total amount of auxin diffusing out of the tip (here expressed as the angle of curvature) is the same in the presence of unilateral light as in darkness (compare Figure A and B). This result indicates that light does not cause the photodestruction of auxin on the illuminated side, as had been proposed by some investigators.

Consistent with both the Cholodny–Went hypothesis and the acid growth hypothesis, the apoplastic pH on the shaded side of a phototropically bending stem or coleoptiles is more acidic than the side facing the light.



Gravitropism Also Involves Lateral Redistribution of Auxin

When dark-grown *Avena* seedlings are oriented horizontally, the coleoptiles bend upward in response to gravity. According to the Cholodny–Went model, auxin in a horizontally oriented coleoptile tip is transported laterally to the lower side, causing the lower side of the coleoptile to grow faster than the upper side. Early experimental evidence indicated that the tip of the coleoptile can perceive gravity and redistribute auxin to the lower side. For example, if coleoptile tips are oriented horizontally, a greater amount of auxin diffuses from the lower half than the upper half.

Tissues below the tip are able to respond to gravity as well. For example, when vertically oriented maize coleoptiles are decapitated by removal of the upper 2 mm of the tip and oriented horizontally, gravitropic bending occurs at a slow rate for several hours even without the tip. Application of IAA to the cut surface restores the rate of bending to normal levels. This finding indicates that both the perception of the gravitational stimulus and the lateral redistribution of auxin can occur in the tissues below the tip, although the tip is still required for auxin production. Lateral redistribution of auxin in shoot apical meristems is more difficult to demonstrate than in coleoptiles because of the presence of leaves. In recent years, molecular markers have been widely used as reporter genes to detect lateral auxin gradients in horizontally placed stems and roots.

In soybean hypocotyls, gravitropism leads to a rapid asymmetry in the accumulation of a group of auxin-stimulated mRNAs called **SAURs** (*s*mall *a*uxin *u*p-regulated *R*NAs**)**. In vertical seedlings, SAUR gene expression is symmetrically distributed. Within 20 minutes after the seedling is oriented horizontally, SAURs begin to accumulate on the lower half of the hypocotyl. Under these conditions, gravitropic bending first becomes evident after 45 minutes, well after the induction of the SAURs. The existence of a lateral gradient in SAUR gene expression is indirect evidence for the existence of a lateral gradient in auxin detectable within 20 minutes of the gravitropic stimulus. The *GH3* gene family is also up-regulated within 5 minutes of auxin treatment and has been used as a molecular marker for the presence of auxin. By fusing an artificial promoter sequence based on the *GH3* promoter to the *GUS* reporter gene, it is possible to visualize the lateral gradient in auxin concentration that occurs during both photo- and gravitropism.

Statoliths Serve as Gravity Sensors in Shoots and Roots

Unlike unilateral light, gravity does not form a gradient between the upper and lower sides of an organ. All parts of the plant experience the gravitational stimulus equally. How do plant cells detect gravity? The only way that gravity can be sensed is through the motion of a falling or sedimenting body.

Obvious candidates for intracellular gravity sensors in plants are the large, dense amyloplasts that are present in many plant cells. These specialized amyloplasts are of sufficiently high density relative to the cytosol that they readily sediment to the bottom of the cell. Amyloplasts that function as gravity sensors are called **statoliths**, and the specialized gravity-sensing cells in which they occur are called **statocytes**. Whether the statocyte is able to detect the downward motion of the statolith as it passes through the cytoskeleton or whether the stimulus is perceived only when the statolith comes to rest at the bottom of the cell has not yet been resolved.

Shoots and Coleoptiles. In shoots and coleoptiles, gravity is perceived in the **starch sheath**, a layer of cells that surrounds the vascular tissues of the shoot. The starch sheath is continuous with the endodermis of the root, but unlike the endodermis it contains amyloplasts. *Arabidopsis* mutants lacking amyloplasts in the starch sheath display agravitropic shoot growth but normal gravitropic root growth.

Auxin Regulates Apical Dominance

In most higher plants, the growing apical bud inhibits the growth of lateral (axillary) buds—a phenomenon called **apical dominance**. Removal of the shoot apex (decapitation) usually results in the growth of one or more of the lateral buds. Not long after the discovery of auxin, it was found that IAA could substitute for the apical bud in maintaining the inhibition of lateral buds of bean (*Phaseolus vulgaris*) plants.

How does auxin from the shoot apex inhibit the growth of lateral buds? Kenneth V. Thimann and Folke Skoog originally proposed that auxin from the shoot apex inhibits the growth of the axillary bud directly—the so-called *direct inhibition model*. According to the model, the optimal auxin concentration for bud growth is low, much lower than the auxin concentration normally found in the stem. The level of auxin normally present in the stem was thought to inhibit the growth of lateral buds.

Other hormones, such as cytokinins and ABA, may be involved. Direct application of cytokinins to axillary buds stimulates bud growth in many species, overriding the inhibitory effect of the shoot apex. Auxin makes the shoot apex a sink for cytokinin synthesized in the root, and this may be one of the factors involved in apical dominance.

Finally, ABA has been found in dormant lateral buds in intact plants. When the shoot apex is removed, the ABA levels in the lateral buds decrease. High levels of IAA in the shoot may help keep ABA levels high in the lateral buds. Removing the apex removes a major source of IAA, which may allow the levels of bud growth inhibitor to fall.

Auxin Promotes the Formation of Lateral and Adventitious Roots

Although elongation of the primary root is inhibited by auxin concentrations greater than 10^{-8} *M*, initiation of lateral (branch) roots and adventitious roots is stimulated by high auxin levels. Lateral roots are commonly found above the elongation and root hair zone and originate from small groups of cells in the pericycle. Auxin stimulates these pericycle cells to divide. The dividing cells gradually form into a root apex, and the lateral root grows through the root cortex and epidermis. Adventitious roots (roots originating from nonroot tissue) can arise in a variety of tissue locations from clusters of mature cells that renew their cell division activity. These dividing cells develop into a root apical meristem in a manner somewhat analogous to the formation of lateral roots. In horticulture, the stimulatory effect of auxin on the formation of adventitious roots has been very useful for the vegetative propagation of plants by cuttings.

IAA is required for at least two steps in the formation of lateral roots:
1. IAA transported acropetally (toward the tip) in the stele is required to initiate cell division in the pericycle.
2. IAA is required to promote cell division and maintain cell viability in the developing lateral root.

Auxin Delays the Onset of Leaf Abscission

The shedding of leaves, flowers, and fruits from the living plant is known as **abscission**. These parts abscise in a region called the **abscission zone**, which is located near the base of the petiole of leaves. In most plants, leaf abscission is preceded by the differentiation of a distinct layer of cells, the **abscission layer**, within the abscission zone. During leaf senescence, the walls of the cells in the abscission layer are digested, which causes them to become soft and weak. The leaf eventually breaks off at the abscission layer as a result of stress on the weakened cell walls. Auxin levels are high in young leaves, progressively decrease in maturing leaves, and are relatively low in senescing leaves when the abscission process begins. The role of auxin in leaf abscission can be readily demonstrated by excision of the blade from a mature leaf, leaving the petiole intact on the stem. Whereas removal of the leaf blade accelerates the formation of the abscission layer in the petiole, application of IAA in lanolin paste to the cut surface of the petiole prevents the formation of the abscission layer. (Lanolin paste alone does not prevent abscission.). These results suggest the following:
• Auxin transported from the blade normally prevents abscission.
• Abscission is triggered during leaf senescence, when auxin is no longer being produced.

Auxin Transport Regulates Floral Bud Development

Treating *Arabidopsis* plants with the auxin transport inhibitor NPA causes abnormal floral development, suggesting that polar auxin transport in the inflorescence meristem is required for normal floral development.

Auxin Promotes Fruit Development

Much evidence suggests that auxin is involved in the regulation of fruit development. Auxin is produced in pollen and in the endosperm and the embryo of developing seeds, and the initial stimulus for fruit growth may result from pollination. Successful pollination initiates ovule growth, which is known as **fruit set**. After fertilization, fruit growth may depend on auxin produced in developing seeds. The endosperm may contribute auxin during the initial stage of fruit growth, and the developing embryo may take over as the main auxin source during the later stages.

Auxin Induces Vascular Differentiation

New vascular tissues differentiate directly below developing buds and young growing leaves, and removal of the young leaves prevents vascular differentiation. The ability of an apical bud to stimulate vascular differentiation can be demonstrated in tissue culture. When the apical bud is grafted onto a clump of undifferentiated cells, or *callus*, xylem and phloem differentiate beneath the graft.

The relative amounts of xylem and phloem formed are regulated by the auxin concentration: High auxin concentrations induce the differentiation of xylem and phloem, but only phloem differentiates at low auxin concentrations. Similarly, experiments on stem tissues have shown that low auxin concentrations induce phloem differentiation, whereas higher IAA levels induce xylem.

The regeneration of vascular tissue following wounding is also controlled by auxin produced by the young leaf directly above the wound site. Removal of the leaf prevents the regeneration of vascular tissue, and applied auxin can substitute for the leaf in stimulating regeneration.

Vascular differentiation is polar and occurs from leaves to roots. In woody perennials, auxin produced by growing buds in the spring stimulates activation of the cambium in a basipetal direction. The new round of secondary growth begins at the smallest twigs and progresses downward toward the root tip.

Synthetic Auxins Have a Variety of Commercial Uses

Auxins have been used commercially in agriculture and horticulture for more than 50 years. The early commercial uses included prevention of fruit and leaf drop, promotion of flowering in pineapple, induction of parthenocarpic fruit, thinning of fruit, and rooting of cuttings for plant propagation. Rooting is enhanced if the excised leaf or stem cutting is dipped in an auxin solution, which increases the initiation of adventitious roots at the cut end. This is the basis of commercial rooting compounds, which consist mainly of a synthetic auxin mixed with talcum powder.

In some plant species, seedless fruits may be produced naturally, or they may be induced by treatment of the unpollinated flowers with auxin. The production of such seedless fruits is called **parthenocarpy**. In stimulating the formation of parthenocarpic fruits, auxin may act primarily to induce fruit set, which in turn may trigger the endogenous production of auxin by certain fruit tissues to complete the developmental process.

Ethylene is also involved in fruit development, and some of the effects of auxin on fruiting may result from the promotion of ethylene synthesis. In addition to these applications, today auxins are widely used as herbicides. The chemicals 2,4-D and dicamba are probably the most widely used synthetic auxins. Synthetic auxins are very effective because they are not metabolized by the plant as quickly as IAA is. Because maize and other monocotyledons can rapidly inactivate synthetic auxins by conjugation, these auxins are used by farmers for the control of dicot weeds, also called *broad-leaved weeds*, in commercial cereal fields, and by home gardeners for the control of weeds such as dandelions and daisies in lawns.

AUXIN SIGNAL TRANSDUCTION PATHWAYS

The ultimate goal of research on the molecular mechanism of hormone action is to reconstruct each step in the signal transduction pathway, from receptor binding to the physiological response. In this last section, we will examine candidates for the auxin receptor and then discuss the various signaling pathways that have been implicated in auxin action. Finally we will turn our attention to auxinregulated gene expression.

ABP1 Functions as an Auxin Receptor:

In addition to its possible direct role in plasma membrane H^+-ATPase activation (discussed earlier), the auxin-binding protein ABP1 appears to function as an auxin receptor in other signal transduction pathways. ABP1 homologs have been identified in a variety of monocot and dicot species. Knockouts of the *ABP1* gene in *Arabidopsis* are lethal, and less severe mutations result in altered development. Recent studies indicate that, despite being localized primarily on the endoplasmic reticulum (ER), a small amount of ABP1 is secreted to the plasma membrane outer surface where it interacts with auxin to cause protoplast swelling and H^+-pumping .

However, it is unlikely that ABP1 mediates all auxin response pathways because expression of a number of auxin-responsive genes is not affected when protoplasts are incubated with anti-ABP1 antibodies. It is also unclear what role the ABP1 in the ER plays in auxin-responsive signal transduction. Finally, it remains to be determined whether ABP57, the soluble and unrelated ABP from rice that activates the H^+-ATPase (see Figure), is involved in a signal transduction pathway.

Calcium and Intracellular pH Are Possible Signaling Intermediates

Calcium plays an important role in signal transduction in animals and is thought to be involved in the action of certain plant hormones as well. The role of calcium in auxin action seems very complex and, at this point in time, very uncertain. Nevertheless, some experimental evidence shows that auxin increases the level of free calcium in the cell. Changes in cytoplasmic pH can also serve as a second messenger in animals and plants. In plants, auxin induces a decrease in cytosolic pH of about 0.2 units within 4 minutes of application. The cause of this pH drop is not known. Since the cytosolic pH is normally around 7.4, and the pH optimum of the plasma membrane H^+-ATPase is 6.5, a decrease in the cytosolic pH of 0.2 units could cause a marked increase in the activity of the plasma membrane H^+-ATPase.

The decrease in cytosolic pH might also account for the auxin-induced increase in free intracellular calcium, by promoting the dissociation of bound forms. MAP kinases that play a role in signal transduction by phosphorylating proteins in a cascade that ultimately activates transcription factors have also been implicated in auxin responses. When tobacco cells are deprived of auxin, they arrest at the end of either the G1 or the G2 phase and cease dividing; if auxin is added back into the culture medium, the cell cycle resumes. Auxin appears to exert its effect on the cell cycle primarily by stimulating the synthesis of the major cyclin-dependent protein kinase (CDK): Cdc2 (*c*ell *d*ivision *c*ycle *2*).

Auxin-Induced Genes Fall into Two Classes: Early and Late

One of the important functions of the signal transduction pathway(s) initiated when auxin binds to its receptor is the activation of a select group of transcription factors. The activated transcription factors enter the nucleus and promote the expression of specific genes. Genes whose expression is stimulated by the activation of preexisting transcription factors are called **primary response genes** or **early genes**.

This definition implies that all of the proteins required for auxin-induced expression of the early genes are present in the cell at the time of exposure to the hormone; thus, early-gene expression cannot be blocked by inhibitors of protein synthesis such as cycloheximide. As a consequence, the time required for the expression of the early genes can be quite short, ranging from a few minutes to several hours.

In general, primary response genes have three main functions:

(1) Some of the early genes encode proteins that regulate the transcription of **secondary response genes**, or **late genes**, that are required for the long-term responses to the hormone. Because late genes require de novo protein synthesis, their expression can be blocked by protein synthesis inhibitors.
(2) Other early genes are involved in intercellular communication, or cell-to-cell signaling.
(3) Another group of early genes is involved in adaptation to stress.

Five major classes of early auxin-responsive genes have been identified:

• **Genes involved in auxin-regulated growth and development:**
1. The *AUX/IAA* gene family
2. The *SAUR* gene family
3. The *GH3* gene family

• **Stress response genes:**
1. Genes encoding glutathione S-transferases
2. Genes encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, the key enzyme in the ethylene biosynthetic pathway

Early genes for growth and development.

Members of the *AUX/IAA* gene family encode short-lived transcription factors that function as repressors or activators of the expression of late auxin-inducible genes. The expression of most of the *AUX/IAA* family of genes is stimulated by auxin within 5 to 60 minutes of hormone addition All the genes encode small hydrophilic polypeptides that have putative DNA-binding motifs similar to those of bacterial repressors. They also have short half-lives (about 7 minutes), indicating that they are turning over rapidly.

The *SAUR* gene family was mentioned earlier in the chapter in relation to tropisms. Auxin stimulates the expression of *SAUR* genes within 2 to 5 minutes of treatment, and the response is insensitive to cycloheximide. The five *SAUR* genes of soybean are clustered together, contain no introns, and encode highly similar polypeptides of unknown function. Because of the rapidity of the response, expression of *SAUR* genes has proven to be a convenient probe for the lateral transport of auxin during photo- and gravitropism.

GH3 early-gene family members, identified in both soybean and *Arabidopsis*, are stimulated by auxin within 5 minutes. Mutations in *Arabidopsis GH3*-like genes result in dwarfism and appear to function in light-regulated auxin responses. Because *GH3* expression is a good reflection of the presence of endogenous auxin, a synthetic *GH3*-based **reporter** gene known as *DR5* is widely used in auxin bioassays.

Early genes for stress adaptations. As mentioned earlier in the chapter, auxin is involved in stress responses, such as wounding. Several genes encoding glutathione-S-transferases (GSTs), a class of proteins stimulated by various stress conditions, are induced by elevated auxin concentrations.

Likewise, ACC synthase, which is also induced by stress and is the rate-limiting step in ethylene biosynthesis, is induced by high levels of auxin.

To be induced, the promoters of the early auxin genes must contain response elements that bind to the transcription factors that become activated in the presence of auxin.

A limited number of these response elements appear to be arranged combinatorily within the promoters of a variety of auxin-induced genes.

Auxin-Responsive Domains Are Composite Structures

Aconserved **auxin response element** (**AuxRE**) within the promoters of the early auxin genes, like *GH3*, is usually combined with other response elements to form **auxin response domains** (**AuxRDs**). For example, the *GH3* gene promoter of soybean is composed of three independently acting AuxRDs (each containing multiple AuxREs) that contribute incrementally to the strong auxin inducibility of the promoter.

Early Auxin Genes Are Regulated by Auxin Response Factors

As noted previously, early auxin genes are by definition insensitive to protein synthesis inhibitors such as cycloheximide. Instead of being inhibited, the expression of many of the early auxin genes has been found to be stimulated by cycloheximide.

Cycloheximide stimulation of gene expression is accomplished both by transcriptional activation and by mRNA stabilization. Transcriptional activation of a gene by inhibitors of protein synthesis usually indicates that the gene is being repressed by a short-lived repressor protein or by a regulatory pathway that involves a protein with a high turnover rate.

A family of **auxin response factors** (**ARFs**) function as transcriptional activators by binding to the auxin response element TGTCTC, which is present in the promoters of *GH3* and other early auxin response genes. Mutations in ARF genes result in severe developmental defects. To bind the AuxRE stably, ARFs must form dimers. It has been proposed that ARF dimers promote transcription by binding to two AuxREs arranged in a palindrome.

Recent studies also indicate that proteins encoded by the *AUX/IAA* gene family (itself one of the early auxin response gene families) can inhibit the transcription of early auxin response genes by forming inactive heterodimers with ARFs.

These inactive heterodimers may act to inhibit ARF–AuxRE binding, thereby blocking either gene activation or repression. AUX/IAA proteins may thus function as ARF inhibitors.

It is now believed that auxin induces the transcription of the early response genes by promoting the proteolytic degradation of the inhibitory AUX/IAA proteins so that active ARF dimers can form. The precise mechanism by which auxin causes AUX/IAA turnover is unknown, although it is known to involve ubiquitination by a ubiquitin ligase and proteolysis by the massive 26S proteasome complex. Note that a negative feedback loop is introduced into the pathway by virtue of the fact that one of the gene families turned on by auxin is *AUX/IAA*, which inhibits the response.

A model for auxin regulation of the early response genes based on the findings described here is shown in Figure.

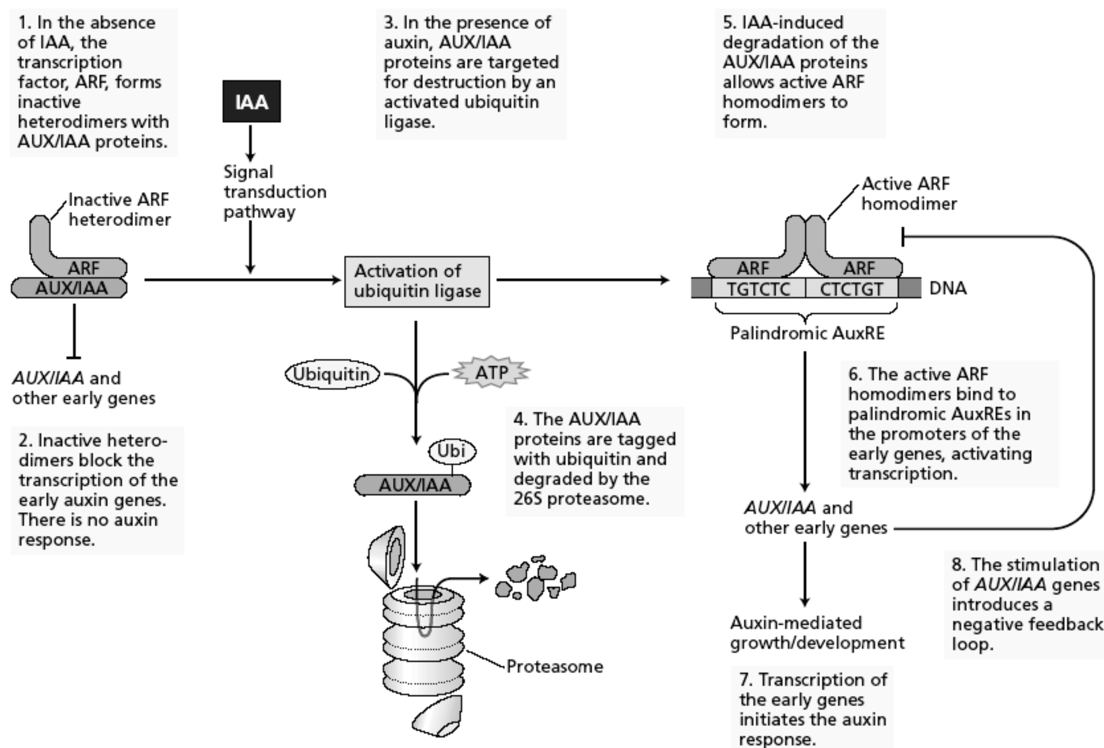


FIGURE A model for auxin regulation of transcriptional activation of early response genes by auxin.

CYTOKININS

The cytokinins were discovered in the search for factors that stimulate plant cells to divide (i.e., undergo cytokinesis). Since their discovery, cytokinins have been shown to have effects on many other physiological and developmental processes, including leaf senescence, nutrient mobilization, apical dominance, the formation and activity of shoot apical meristems, floral development, the breaking of bud dormancy, and seed germination. Cytokinins also appear to mediate many aspects of light-regulated development, including chloroplast differentiation, the development of autotrophic metabolism, and leaf and cotyledon expansion.

Although cytokinins regulate many cellular processes, the control of cell division is central in plant growth and development and is considered diagnostic for this class of plant growth regulators.

CELL DIVISION AND PLANT DEVELOPMENT

Plant cells form as the result of cell divisions in a primary or secondary meristem. Newly formed plant cells typically enlarge and differentiate, but once they have assumed their function—whether transport, photosynthesis, support, storage, or protection—usually they do not divide again during the life of the plant. In this respect they appear to be similar to animal cells, which are considered to be terminally differentiated.

However, this similarity to the behavior of animal cells is only superficial. Almost every type of plant cell that retains its nucleus at maturity

Differentiated Plant Cells Can Resume Division

Under some circumstances, mature, differentiated plant cells may resume cell division in the intact plant. In many species, mature cells of the cortex and/or phloem resume division to form secondary meristems, such as the vascular cambium or the cork cambium. The abscission zone at the base of a leaf petiole is a region where mature parenchyma cells begin to divide again after a period of mitotic inactivity, forming a layer of cells with relatively weak cell walls where abscission can occur.

Wounding of plant tissues induces cell divisions at the wound site. Even highly specialized cells, such as phloem fibers and guard cells, may be stimulated by wounding to divide at least once. Wound-induced mitotic activity typically is self-limiting; after a few divisions the derivative cells stop dividing and redifferentiate. However, when the soildwelling bacterium *Agrobacterium tumefaciens* invades a wound, it can cause the neoplastic (tumor-forming) disease known as **crown gall**. This phenomenon is dramatic natural evidence of the mitotic potential of mature plant cells.

Without *Agrobacterium* infection, the wound-induced cell division would subside after a few days and some of the new cells would differentiate as a protective layer of cork cells or vascular tissue. However, *Agrobacterium* changes the character of the cells that divide in response to the wound, making them tumorlike. They do not stop dividing; rather they continue to divide throughout the life of the plant to produce an unorganized mass of tumorlike tissue called a **gall**.

Diffusible Factors May Control Cell Division

The considerations addressed in the previous section suggest that mature plant cells stop dividing because they no longer receive a particular signal, possibly a hormone, that is necessary for the initiation of cell division. The idea that cell division may be initiated by a diffusible factor originated with the Austrian plant physiologist G. Haberlandt, who, in about 1913, demonstrated that vascular tissue contains a water-soluble substance or substances that will stimulate the division of wounded potato tuber tissue. The effort to determine the nature of this factor (or factors) led to the discovery of the cytokinins in the 1950s.

THE DISCOVERY, IDENTIFICATION, AND PROPERTIES OF CYTOKININS

A great many substances were tested in an effort to initiate and sustain the proliferation of normal stem tissues in culture. Materials ranging from yeast extract to tomato juice were found to have a positive effect, at least with some tissues. However, culture growth was stimulated most dramatically when the liquid endosperm of coconut, also known as coconut milk, was added to the culture medium.

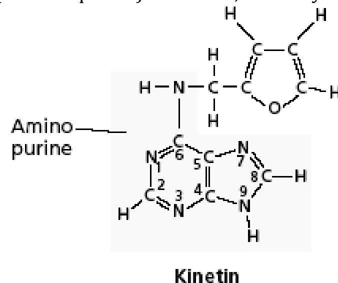
Philip White's nutrient medium, supplemented with an auxin and 10 to 20% coconut milk, will support the continued cell division of mature, differentiated cells from a wide variety of tissues and species, leading to the formation of callus tissue . This finding indicated that coconut milk contains a substance or substances that stimulate mature cells to enter and remain in the cell division cycle.

Eventually coconut milk was shown to contain the cytokinin *zeatin*, but this finding was not obtained until several years after the discovery of the cytokinins. The first cytokinin to be discovered was the synthetic analog kinetin.

Kinetin Was Discovered as a Breakdown Product of DNA

In the 1940s and 1950s, Folke Skoog and coworkers at the University of Wisconsin tested many substances for their ability to initiate and sustain the proliferation of cultured tobacco pith tissue. They had observed that the nucleic acid base adenine had a slight promotive effect, so they tested the possibility that nucleic acids would stimulate division in this tissue. Surprisingly, autoclaved herring sperm DNA had a powerful cell division–promoting effect.

After much work, a small molecule was identified from the autoclaved DNA and named **kinetin**. It was shown to be an adenine (or aminopurine) derivative, 6-furfurylaminopurine



Kinetin

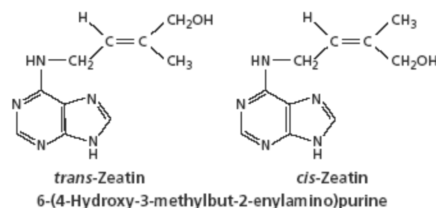
In the presence of an auxin, kinetin would stimulate tobacco pith parenchyma tissue to proliferate in culture. No kinetin-induced cell division occurs without auxin in the culture medium.

Kinetin is not a naturally occurring plant growth regulator, and it does not occur as a base in the DNA of any species. It is a by-product of the heat-induced degradation of DNA, in which the deoxyribose sugar of adenosine is converted to a furfuryl ring and shifted from the 9 position to the 6 position on the adenine ring.

The discovery of kinetin was important because it demonstrated that cell division could be induced by a simple chemical substance. Of greater importance, the discovery of kinetin suggested that naturally occurring molecules with structures similar to that of kinetin regulate cell division activity within the plant. This hypothesis proved to be correct.

Zeatin Is the Most Abundant Natural Cytokinin

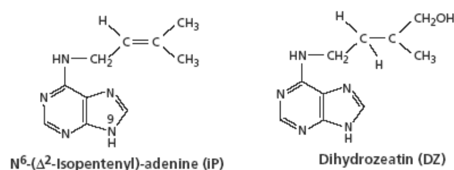
Several years after the discovery of kinetin, extracts of the immature endosperm of corn (*Zea mays*) were found to contain a substance that has the same biological effect as kinetin. This substance stimulates mature plant cells to divide when added to a culture medium along with an auxin. Letham (1973) isolated the molecule responsible for this activity and identified it as *trans*-6-(4-hydroxy-3- methylbut-2-enylamino)purine, which he called **zeatin**:



The molecular structure of zeatin is similar to that of kinetin. Both molecules are adenine or aminopurine derivatives. Although they have different side chains, in both cases the side chain is attached to the 6 nitrogen of the aminopurine. Because the side chain of zeatin has a double bond, it can exist in either the *cis* or the *trans* configuration.

In higher plants, zeatin occurs in both the *cis* and the *trans* configurations, and these forms can be interconverted by an enzyme known as *zeatin isomerase*. Although the *trans* form of zeatin is much more active in biological assays, the *cis* form may also play important roles, as suggested by the fact that it has been found in high levels in a number of plant species and particular tissues. Agene encoding a glucosyl transferase enzyme specific to *cis*-zeatin has recently been cloned, which further supports a biological role for this isoform of zeatin.

Since its discovery in immature maize endosperm, zeatin has been found in many plants and in some bacteria. It is the most prevalent cytokinin in higher plants, but other substituted aminopurines that are active as cytokinins have been isolated from many plant and bacterial species. These aminopurines differ from zeatin in the nature of the side chain attached to the 6 nitrogen or in the attachment of a side chain to carbon 2:

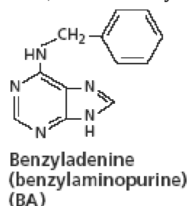


In addition, these cytokinins can be present in the plant as a **riboside** (in which a ribose sugar is attached to the 9 nitrogen of the purine ring), a **ribotide** (in which the ribose sugar moiety contains a phosphate group), or a **glycoside** (in which a sugar molecule is attached to the 3, 7, or 9 nitrogen of the purine ring, or to the oxygen of the zeatin or dihydrozeatin side chain).

Some Synthetic Compounds Can Mimic or Antagonize Cytokinin Action

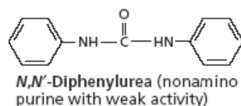
Cytokinins are defined as compounds that have biological activities similar to those of *trans*-zeatin. These activities include the ability to do the following:
• Induce cell division in callus cells in the presence of an auxin
• Promote bud or root formation from callus cultures when in the appropriate molar ratios to auxin
• Delay senescence of leaves
• Promote expansion of dicot cotyledons

Many chemical compounds have been synthesized and tested for cytokinin activity. Analysis of these compounds provides insight into the structural requirements for activity. Nearly all compounds active as cytokinins are N6-substituted aminopurines, such as benzyladenine (BA):

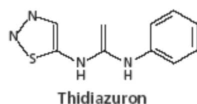


Benzyladenine (benzylaminopurine) (BA)

and all the naturally occurring cytokinins are aminopurine derivatives. There are also synthetic cytokinin compounds that have not been identified in plants, most notable of which are the diphenylurea-type cytokinins, such as thidiazuron, which is used commercially as a defoliant and an herbicide:

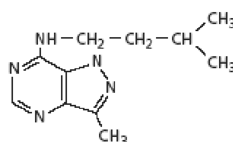


N,N′-**Diphenylurea** (nonamino purine with weak activity)



Thidiazuron

In the course of determining the structural requirements for cytokinin activity, investigators found that some molecules act as *cytokinin antagonists*:



3-Methyl-7-(3-methylbutylamino)pyrazolo[4,3-D]pyrimidine

Cytokinins Occur in Both Free and Bound Forms

Hormonal cytokinins are present as free molecules (not covalently attached to any macromolecule) in plants and certain bacteria. Free cytokinins have been found in a wide spectrum of angiosperms and probably are universal in this group of plants. They have also been found in algae, diatoms, mosses, ferns, and conifers.

The regulatory role of cytokinins has been demonstrated only in angiosperms, conifers, and mosses, but they may function to regulate the growth, development, and metabolism of all plants. Usually zeatin is the most abundant naturally occurring free cytokinin, but *dihydrozeatin* (*DZ*) and *isopentenyl adenine* (*iP*) also are commonly found in higher plants and bacteria. Numerous derivatives of these three cytokinins have been identified in plant extract.

Transfer RNA (tRNA) contains not only the four nucleotides used to construct all other forms of RNA, but also some unusual nucleotides in which the base has been modified. Some of these "hypermodified" bases act as cytokinins when the tRNA is hydrolyzed and tested in one of the cytokinin bioassays. Some plant tRNAs contain *cis*-zeatin as a hypermodified base. However, cytokinins are not confined to plant tRNAs. They are part of certain tRNAs from all organisms, from bacteria to humans.

The Hormonally Active Cytokinin Is the Free Base

It has been difficult to determine which species of cytokinin represents the active form of the hormone, but the recent identification of the cytokinin receptor CRE1 has allowed this question to be addressed. The relevant experiments have shown that the free-base form of *trans*-zeatin, but not its riboside or ribotide derivatives, binds directly to CRE1, indicating that the free base is the active form.

Although the free-base form of *trans*-zeatin is thought to be the hormonally active cytokinin, some other compounds have cytokinin activity, either because they are readily converted to zeatin, dihydrozeatin, or isopentenyl adenine, or because they release these compounds from other molecules, such as cytokinin glucosides. For example, tobacco cells in culture do not grow unless cytokinin ribosides supplied in the culture medium are converted to the free base.

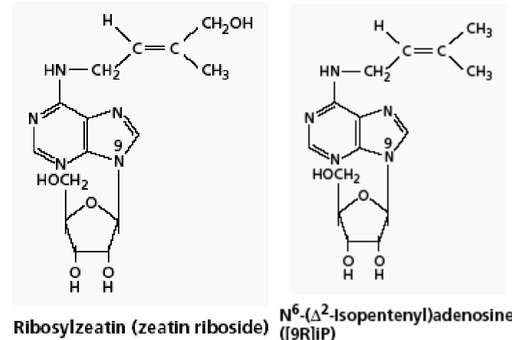
In another example, excised radish cotyledons grow when they are cultured in a solution containing the cytokinin base benzyladenine (BA, an N6-substituted aminopurine cytokinin). The cultured cotyledons readily take up the hormone and convert it to various BA glucosides, BA ribonucleoside, and BA ribonucleotide. When the cotyledons are transferred back to a medium lacking a cytokinin, their growth rate declines, as do the concentrations of BA, BA ribonucleoside, and BA ribonucleotide in the tissues.

However, the level of the BA glucosides remains constant. This finding suggests that the glucosides cannot be the active form of the hormone.

Some Plant Pathogenic Bacteria, Insects, and Nematodes Secrete Free Cytokinins

Some bacteria and fungi are intimately associated with higher plants. Many of these microorganisms produce and secrete substantial amounts of cytokinins and/or cause the plant cells to synthesize plant hormones, including cytokinins. The cytokinins produced by microorganisms include *trans*-zeatin, [9R]iP, *cis*-zeatin, and their ribosides. Infection of plant tissues with these microorganisms can induce the tissues to divide and, in some cases, to form special structures, such as mycorrhizae, in which the microorganism can reside in a mutualistic relationship with the plant.

In addition to the crown gall bacterium, *Agrobacterium tumefaciens*, other pathogenic bacteria may stimulate plant cells to divide. For example, *Corynebacterium fascians* is a major cause of the growth abnormality known as **witches'-broom**. The shoots of plants infected by *C. fascians* resemble an old-fashioned straw broom because the lateral buds, which normally remain dormant, are stimulated by the bacterial cytokinin to grow.



Ribosylzeatin (zeatin riboside) N^6-(Δ^2-Isopentenyl)adenosine ([9R]iP)

Infection with a close relative of the crown gall organism, *Agrobacterium rhizogenes*, causes masses of roots instead of callus tissue to develop from the site of infection. *A. rhizogenes* is able to modify cytokinin metabolism in infected plant tissues.

Certain insects secrete cytokinins, which may play a role in the formation of galls utilized by these insects as feeding sites. Root-knot nematodes also produce cytokinins, which may be involved in manipulating host development to produce the giant cells from which the nematode feeds.

BIOSYNTHESIS, METABOLISM, AND TRANSPORT OF CYTOKININS

The side chains of naturally occurring cytokinins are chemically related to rubber, carotenoid pigments, the plant hormones gibberellin and abscisic acid, and some of the plant defense compounds known as phytoalexins. All of these compounds are constructed, at least in part, from isoprene units.

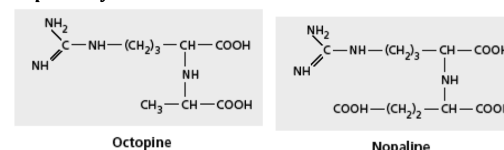
Isoprene is similar in structure to the side chains of zeatin and iP. These cytokinin side chains are synthesized from an isoprene derivative. Large molecules of rubber and the carotenoids are constructed by the polymerization of many isoprene units; cytokinins contain just one of these units. The precursor(s) for the formation of these isoprene structures are either mevalonic acid or pyruvate plus 3-phosphoglycerate, depending on which pathway is involved. These precursors are converted to the biological isoprene unit dimethylallyl diphosphate (DMAPP).

Crown Gall Cells Have Acquired a Gene for Cytokinin Synthesis

Bacteria-free tissues from crown gall tumors proliferate in culture without the addition of any hormones to the culture medium. Crown gall tissues contain substantial amounts of both auxin and free cytokinins. Furthermore, when radioactively labeled adenine is fed to periwinkle (*Vinca rosea*) crown gall tissues, it is incorporated into both zeatin and zeatin riboside, demonstrating that gall tissues contain the cytokinin biosynthetic pathway. Control stem tissue, which has not been transformed by *Agrobacterium*, does not incorporate labeled adenine into cytokinins.

During infection by *Agrobacterium tumefaciens*, plant cells incorporate bacterial DNA into their chromosomes. The virulent strains of *Agrobacterium* contain a large plasmid known as the **Ti plasmid**. Plasmids are circular pieces of extrachromosomal DNA that are not essential for the life of the bacterium. However, plasmids frequently contain genes that enhance the ability of the bacterium to survive in special environments.

A small portion of the Ti plasmid, known as the **TDNA**, is incorporated into the nuclear DNA of the host plant cell. T-DNA carries genes necessary for the biosynthesis of *trans*-zeatin and auxin, as well as a member of a class of unusual nitrogencontaining compounds called *opines* . Opines are not synthesized by plants except after crown gall transformation. The T-DNA gene involved in cytokinin biosynthesis—known as the *ipt*1 gene—encodes an ***i**so**p**entenyl **t**ransferase*



Octopine Nopaline

(IPT) enzyme that transfers the isopentenyl group from DMAPP to AMP (adenosine monophosphate) to form isopentenyl adenine ribotide. The *ipt* gene has been called the *tmr* locus because, when *inactivated* by mutation, it results in "rooty" tumors. Isopentenyl adenine ribotide can be converted to the active cytokinins isopentenyl adenine, *trans*zeatin, and dihydrozeatin by endogenous enzymes in plant cells. This conversion route is similar to the pathway for cytokinin synthesis that has been postulated for normal tissue.

The T-DNA also contains two genes encoding enzymes that convert tryptophan to the auxin indole-3-acetic acid (IAA). This pathway of auxin biosynthesis differs from the one in nontransformed cells and involves indoleacetamide as an intermediate. The *ipt* gene and the two auxin biosynthetic enes of T-DNA are **phyto-oncogenes**, since they can induce tumors in plants..

Because their promoters are plant eukaryotic promoters, none of the T-DNAgenes are expressed in the bacterium; rather they are transcribed after they are inserted into the plant chromosomes. Transcription of the genes leads to synthesis of the enzymes they encode, resulting in the production of zeatin, auxin, and an opine. The bacterium can utilize the opine as a nitrogen source, but cells of higher plants cannot. Thus, by transforming the plant cells, the bacterium provides itself with an expanding environment (the gall tissue) in which the host cells are directed to produce a substance (the opine) that only the bacterium can utilize for its nutrition .

An important difference between the control of cytokinin biosynthesis in crown gall tissues and in normal tissues is that the T-DNA genes for cytokinin synthesis are expressed in all infected cells, even those in which the native plant genes for biosynthesis of the hormone are normally repressed.

IPT Catalyzes the First Step in Cytokinin Biosynthesis

The first committed step in cytokinin biosynthesis is the transfer of the isopentenyl group of dimethylallyl diphosphate (DMAPP) to an adenosine moiety. An enzyme

that catalyzes such an activity was first identified in the cellular slime mold *Dictyostelium discoideum*, and subsequently the *ipt* gene from *Agrobacterium* was found to encode such an enzyme. In both cases, DMAPP and AMP are converted to isopentenyladenosine-5'-monophosphate (iPMP). As noted earlier, cytokinins are also present in the tRNAs of most cells, including plant and animal cells. The tRNA cytokinins are synthesized by modification of specific adenine residues within the fully transcribed tRNA. As with the free cytokinins, isopentenyl groups are transferred to the adenine molecules from DMAPP by an enzyme call tRNA-IPT. The genes for tRNA-IPT have been cloned from many species.

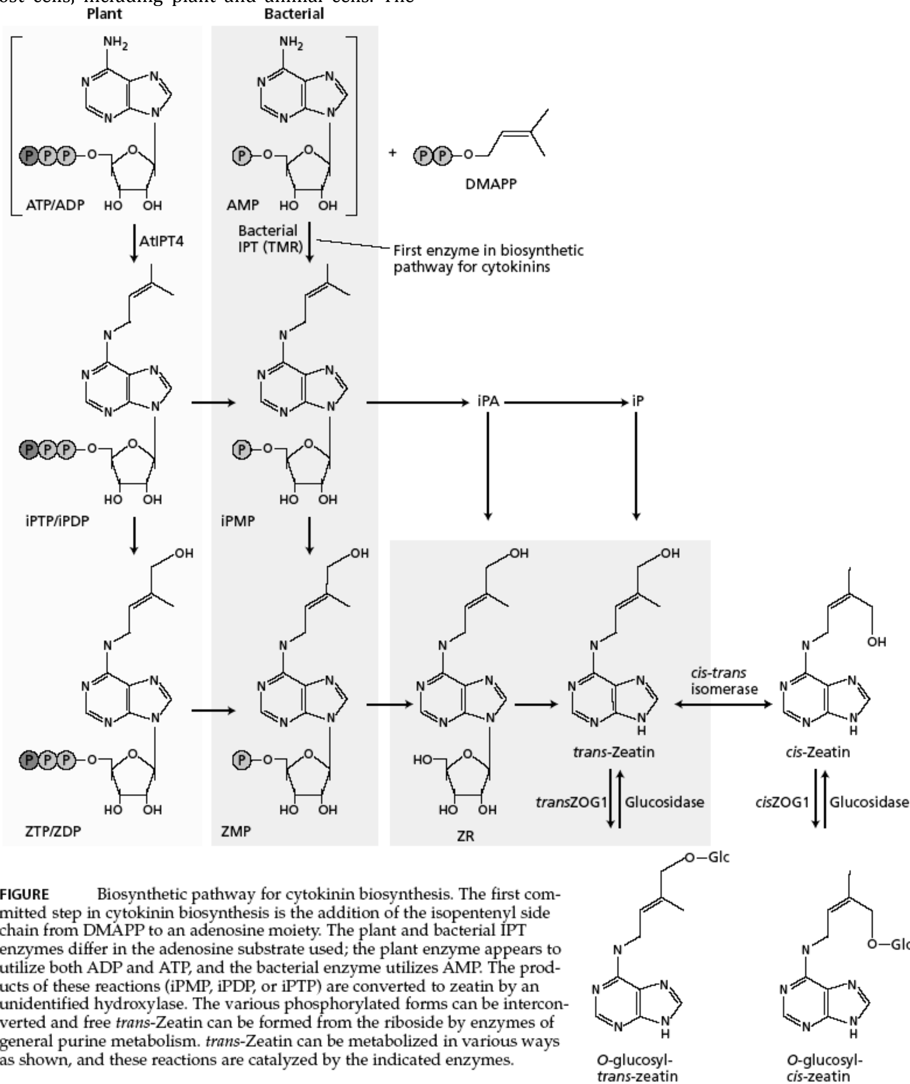


FIGURE Biosynthetic pathway for cytokinin biosynthesis. The first committed step in cytokinin biosynthesis is the addition of the isopentenyl side chain from DMAPP to an adenosine moiety. The plant and bacterial IPT enzymes differ in the adenosine substrate used; the plant enzyme appears to utilize both ADP and ATP, and the bacterial enzyme utilizes AMP. The products of these reactions (iPMP, iPDP, or iPTP) are converted to zeatin by an unidentified hydroxylase. The various phosphorylated forms can be interconverted and free *trans*-Zeatin can be formed from the riboside by enzymes of general purine metabolism. *trans*-Zeatin can be metabolized in various ways as shown, and these reactions are catalyzed by the indicated enzymes.

The possibility that free cytokinins are derived from tRNA has been explored extensively. Although the tRNA bound cytokinins can act as hormonal signals for plant cells if the tRNA is degraded and fed back to the cells, it is unlikely that any significant amount of the free hormonal cytokinin in plants is derived from the turnover of tRNA.

An enzyme with IPT activity was identified from crude extracts of various plant tissues, but researchers were unable to purify the protein to homogeneity. Recently, plant *IPT* genes were cloned after the *Arabidopsis* genome was analyzed for potential *ipt*-like sequence. Nine different *IPT* genes were identified

Cytokinins from the Root Are Transported to the Shoot via the Xylem

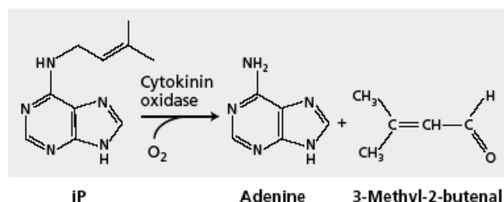
Root apical meristems are major sites of synthesis of the free cytokinins in whole plants. The cytokinins synthesized in roots appear to move through the xylem into the shoot, along with the water and minerals taken up by the roots. This pathway of cytokinin movement has been inferred from the analysis of xylem exudate.
Roots are not the only parts of the plant capable of synthesizing cytokinins. For example, young maize embryos synthesize cytokinins, as do young developing leaves, young fruits, and possibly many other tissues. Clearly, further studies will be needed to resolve the roles of cytokinins transported from the root versus cytokinins synthesized in the shoot.

A Signal from the Shoot Regulates the Transport of Zeatin Ribosides from the Root

The cytokinins in the xylem exudate are mainly in the form of zeatin ribosides. Once they reach the leaves, some of these nucleosides are converted to the free-base form or to glucosides. Cytokinin glucosides may accumulate to high levels in seeds and in leaves, and substantial amounts may be present even in senescing leaves. Although the glucosides are active as cytokinins in bioassays, often they lack hormonal activity after they form within cells, possibly because they are compartmentalized in such a way that they are unavailable. Evidence from grafting experiments with mutants suggests that the transport of zeatin riboside from the root to the shoot is regulated by signals from the shoot.

Cytokinins Are Rapidly Metabolized by Plant Tissues

Free cytokinins are readily converted to their respective nucleoside and nucleotide forms. Such interconversions likely involve enzymes common to purine metabolism. Many plant tissues contain the enzyme **cytokinin oxidase**, which cleaves the side chain from zeatin (both *cis* and *trans*), zeatin riboside, iP, and their *N*-glucosides, but not their *O*-glucoside derivatives (Figure).



However, dihydrozeatin and its conjugates are resistant to cleavage. Cytokinin oxidase irreversibly inactivates cytokinins, and it could be important in regulating or limiting cytokinin effects. The activity of the enzyme is induced by high cytokinin concentrations, due at least in part to an elevation of the RNA levels for a subset of the genes.

Cytokinin levels can also be regulated by conjugation of the hormone at various positions. The nitrogens at the 3, 7, and 9 positions of the adenine ring of cytokinins can be conjugated to glucose residues. Alanine can also be conjugated to the nitrogen at the 9 positon, forming lupinic acid. These modifications are generally irreversible, and such conjugated forms of cytokinin are inactive in bioassays, with the exception of the N3-glucosides.

The hydroxyl group of the side chain of cytokinins is also the target for conjugation to glucose residues, or in some cases xylose residues, yielding *O*-glucoside and *O*xyloside cytokinins. *O*-glucosides are resistant to cleavage by cytokinin oxidases, which may explain why these derivatives have higher biological activity in some assays than their corresponding free bases have.

The conjugations at the side chain can be removed by glucosidase enzymes to yield free cytokinins, which, as discussed earlier, are the active forms. Thus, cytokinin glucosides may be a storage form, or metabolically inactive state, of these compounds. Agene encoding a glucosidase that can release cytokinins from sugar conjugates has been cloned from maize, and its expression could play an important role in the germination of maize seeds.

Dormant seeds often have high levels of cytokinin glucosides but very low levels of hormonally active free cytokinins. Levels of free cytokinins increase rapidly, however, as germination is initiated, and this increase in free cytokinins is accompanied by a corresponding decrease in cytokinin glucosides.

THE BIOLOGICAL ROLES OF CYTOKININS

Although discovered as a cell division factor, cytokinins can stimulate or inhibit a variety of physiological, metabolic, biochemical, and developmental processes when they are applied to higher plants, and it is increasingly clear that endogenous cytokinins play an important role in the regulation of these events in the intact plant.

In this section we will survey some of the diverse effects of cytokinin on plant growth and development, including a discussion of its role in regulating cell division. The discovery of the tumor-inducing Ti plasmid in the plant-pathogenic bacterium *Agrobacterium tumefaciens* provided plant scientists with a powerful new tool for introducing foreign genes into plants, and for studying the role of cytokinin in development. In addition to its role in cell proliferation, cytokinin affects many other processes, including differentiation, apical dominance, and senescence.

Cytokinins Regulate Cell Division in Shoots and Roots

As discussed earlier, cytokinins are generally required for cell division of plant cells in vitro. Several lines of evidence suggest that cytokinins also play key roles in the regulation of cell division in vivo.

Much of the cell division in an adult plant occurs in the meristems. Localized expression of the *ipt* gene of *Agrobacterium* in somatic sectors of tobacco leaves causes the formation of ectopic (abnormally located) meristems, indicating that elevated levels of cytokinin are sufficient to initiate cell divisions in these leaves Overexpression of several of the *Arabidopsis* cytokinin oxidase genes in tobacco results in a reduction of endogenous cytokinin levels and a consequent strong retardation of shoot development due to a reduction in the rate of cell proliferation in the shoot apical meristem. This finding strongly supports the notion that endogenous cytokinins regulate cell division in vivo.

Surprisingly, the same overexpression of cytokinin oxidase in tobacco led to an *enhancement* of root growth, primarily by increasing the size of the root apical meristem. Since the root is a major source of cytokinin, this result may indicate that cytokinins play opposite roles in regulating cell proliferation in root and shoot meristems.

Cytokinins Regulate Specific Components of the Cell Cycle

Cytokinins regulate cell division by affecting the controls that govern the passage of the cell through the cell division cycle. Zeatin levels were found to peak in synchronized culture tobacco cells at the end of S phase, mitosis, and G1 phase. Cytokinins were discovered in relation to their ability to stimulate cell division in tissues supplied with an optimal level of auxin. Evidence suggests that both auxin and cytokinins participate in regulation of the cell cycle and that they do so by controlling the activity of cyclin-dependent kinases. C*yclin-dependent protein kinases* (*CDKs*), in concert with their regulatory subunits, the *cyclins*, are enzymes that regulate the eukaryotic cell cycle. The expression of the gene that encodes the major CDK, Cdc2 (*c*ell *d*ivision *c*ycle *2*), is

regulated by auxin. In pea root tissues, *CDC2* mRNA was induced within 10 minutes after treatment with auxin, and high levels of CDK are induced in tobacco pith when it is cultured on medium containing auxin . However, the CDK induced by auxin is enzymatically inactive, and high levels of CDK alone are not sufficient to permit cells to divide.

Cytokinin has been linked to the activation of a Cdc25- like phosphatase, whose role is to remove an inhibitory phosphate group from the Cdc2 kinase .

This action of cytokinin provides one potential link between cytokinin and auxin in regulating the cell cycle. Recently, a second major input for cytokinin in regulating the cell cycle has emerged. Cytokinins elevate the expression of the *CYCD3* gene, which encodes a *D-type cyclin*. In animal cells, D-type cyclins are regulated by a wide variety of growth factors and play a key role in regulating the passage through the restriction point of the cell cycle in G1. D-type cyclins are thus key players in the regulation of cell proliferation.

The Auxin: Cytokinin Ratio Regulates Morphogenesis in Cultured Tissues

Shortly after the discovery of kinetin, it was observed that the differentiation of cultured callus tissue derived from tobacco pith segments into either roots or shoots depends on the ratio of auxin to cytokinin in the culture medium.

Whereas high auxin:cytokinin ratios stimulated the formation of roots, low auxin:cytokinin ratios led to the formation of shoots. At intermediate levels the tissue grew as an undifferentiated callus.

The effect of auxin: cytokinin ratios on morphogenesis can also be seen in crown gall tumors by mutation of the T-DNA of the *Agrobacterium* Ti plasmid. Mutating the *ipt* gene (the *tmr* locus) of the Ti plasmid blocks zeatin biosynthesis in the infected cells. The resulting high auxin:cytokinin ratio in the tumor cells causes the proliferation of roots instead of undifferentiated callus tissue. In contrast, mutating either of the genes for auxin biosynthesis (*tms* locus) lowers the auxin:cytokinin ratio and stimulates the proliferation of shoots. These partially differentiated tumors are known as teratomas.

Cytokinins Modify Apical Dominance and Promote Lateral Bud Growth

One of the primary determinants of plant form is the degree of apical dominance. Plants with strong apical dominance, such as maize, have a single growing axis with few lateral branches. In contrast, many lateral buds initiate growth in shrubby plants.

Although apical dominance may be determined primarily by auxin, physiological studies indicate that cytokinins play a role in initiating the growth of lateral buds. For example, direct applications of cytokinins to the axillary buds of many species stimulate cell division activity and growth of the buds.
The phenotypes of cytokinin-overproducing mutants are consistent with this result. Wild-type tobacco shows strong apical dominance during vegetative development, and the lateral buds of cytokinin overproducers grow vigorously, developing into shoots that compete with the main shoot. Consequently, cytokinin-overproducing plants tend to be bushy.

Cytokinins Induce Bud Formation in a Moss

Thus far we have restricted our discussion of plant hormones to the angiosperms. However, many plant hormones are present and developmentally active in representative species throughout the plant kingdom. The moss *Funaria hygrometrica* is a well-studied example. The germination of moss spores gives rise to a filament of cells called a *protonema* (plural *protonemata*). The protonema elongates and undergoes cell divisions at the tip, and it forms branches some distance back from the tip.

The transition from filamentous growth to leafy growth begins with the formation of a swelling or protuberance near the apical ends of specific cells. An asymmetric cell division follows, creating the **initial cell**. The initial cell then divides mitotically to produce the **bud**, the structure that gives rise to the leafy gametophyte. During normal growth, buds and branches are regularly initiated, usually beginning at the third cell from the tip of the filament. Light, especially red light, is required for bud formation in *Funaria*. In the dark, buds fail to develop, but cytokinin added to the medium can substitute for the light requirement.

Cytokinin not only stimulates normal bud development; it also increases the total number of buds. Even very low levels of cytokinin (picomolar, or 10^{-12} *M*) can stimulate the first step in bud formation: the swelling at the apical end of the specific protonemal cell.

Cytokinin Overproduction Has Been Implicated in Genetic Tumors

Many species in the genus *Nicotiana* can be crossed to generate interspecific hybrids. More than 300 such interspecific hybrids have been produced; 90% of these hybrids are normal, exhibiting phenotypic characteristics intermediate between those of both parents. The plant used for cigarette tobacco, *Nicotiana tabacum*, for example, is an interspecific hybrid. However, about 10% of these interspecific crosses result in progeny that tend to form spontaneous tumors called **genetic tumors**.

Genetic tumors are similar morphologically to those induced by *Agrobacterium tumefaciens*, discussed at the beginning of this chapter, but genetic tumors form spontaneously in the absence of any external inducing agent. The tumors are composed of masses of rapidly proliferating cells in regions of the plant that ordinarily would contain few dividing cells. Furthermore, the cells divide without differentiating into the cell types normally associated with the tissues giving rise to the tumor. *Nicotiana* hybrids that produce genetic tumors have abnormally high levels of both auxin and cytokinins. Typically, the cytokinin levels in tumor-prone hybrids are five to six times higher than those found in either parent.

Cytokinins Delay Leaf Senescence

Leaves detached from the plant slowly lose chlorophyll, RNA, lipids, and protein, even if they are kept moist and provided with minerals. This programmed aging process leading to death is termed **senescence**. Leaf senescence is more rapid in the dark than in the light. Treating isolated leaves of many species with cytokinins will delay their senescence.

Although applied cytokinins do not prevent senescence completely, their effects can be dramatic, particularly when the cytokinin is sprayed directly on the intact plant. If only one leaf is treated, it remains green after other leaves of

similar developmental age have yellowed and dropped off the plant. Even a small spot on a leaf will remain green if treated with a cytokinin, after the surrounding tissues on the same leaf begin to senesce.

Unlike young leaves, mature leaves produce little if any cytokinin. Mature leaves may depend on root-derived cytokinins to postpone their senescence. Senescence is initiated in soybean leaves by seed maturation—a phenomenon known as *monocarpic senescence*—and can be delayed by seed removal. Although the seedpods control the onset of senescence, they do so by controlling the delivery of root-derived cytokinins to the leaves.

The cytokinins involved in delaying senescence are primarily zeatin riboside and dihydrozeatin riboside, which may be transported into the leaves from the roots through the xylem, along with the transpiration stream.

Cytokinins Promote Movement of Nutrients

Cytokinins influence the movement of nutrients into leaves from other parts of the plant, a phenomenon known as *cytokinin-induced nutrient mobilization*. This process is revealed when nutrients (sugars, amino acids, and so on) radiolabeled with ^{14}C or ^{3}H are fed to plants after one leaf or part of a leaf is treated with a cytokinin. Later the whole plant is subjected to autoradiography to reveal the pattern of movement and the sites at which the labeled nutrients accumulate.

Experiments of this nature have demonstrated that nutrients are preferentially transported to, and accumulated in, the cytokinin-treated tissues. It has been postulated that the hormone causes nutrient mobilization by creating a new source–sink relationship. As discussed in Chapter 10, nutrients translocated in the phloem move from a site of production or storage (the source) to a site of utilization (the sink). The metabolism of the treated area may be stimulated by the hormone so that nutrients move toward it. However, it is not necessary for the nutrient itself to be metabolized in the sink cells because even nonmetabolizable substrate analogs are mobilized by cytokinins.

Cytokinins Promote Chloroplast Development

Although seeds can germinate in the dark, the morphology of dark-grown seedlings is very different from that of lightgrown seedlings: Dark-grown seedlings are said to be **etiolated**. The hypocotyl and internodes of etiolated seedlings are more elongated, cotyledons and leaves do not expand, and chloroplasts do not mature. Instead of maturing as chloroplasts, the proplastids of dark-grown seedlings develop into **etioplasts**, which do not synthesize chlorophyll or most of the enzymes and structural proteins required for the formation of the chloroplast thylakoid system and photosynthesis machinery.

When seedlings germinate in the light, chloroplasts mature directly from the proplastids present in the embryo, but etioplasts also can mature into chloroplasts when etiolated seedlings are illuminated.
If the etiolated leaves are treated with cytokinin before being illuminated, they form chloroplasts with more extensive grana, and chlorophyll and photosynthetic enzymes are synthesized at a greater rate upon illumination. These results suggest that cytokinins—along with other factors, such as light, nutrition, and development— regulate the synthesis of photosynthetic pigments and proteins.

The ability of exogenous cytokinin to enhance de-etiolation of dark-grown seedlings is mimicked by certain mutations that lead to cytokinin overproduction.

Cytokinins Promote Cell Expansion in Leaves and Cotyledons

The promotion of cell enlargement by cytokinins is most clearly demonstrated in the cotyledons of dicots with leafy cotyledons, such as mustard, cucumber, and sunflower. The cotyledons of these species expand as a result of cell enlargement during seedling growth. Cytokinin treatment promotes additional cell expansion, with no increase in the dry weight of the treated cotyledons.

Leafy cotyledons expand to a much greater extent when the seedlings are grown in the light than in the dark, and cytokinins promote cotyledon growth in both light- and dark-grown seedlings. As with auxin- induced growth, cytokinin-stimulated expansion of radish cotyledons is associated with an increase in the mechanical extensibility of the cell walls. However, cytokinin-induced wall loosening is not accompanied by proton extrusion. Neither auxin nor gibberellin promotes cell expansion in cotyledons.

Cytokinins Regulate Growth of Stems and Roots

Although endogenous cytokinins are clearly required for normal cell proliferation in the apical meristem, and therefore normal shoot growth, applied cytokinins typically inhibit the process of cell elongation in both stems and roots. For example, exogenous cytokinin inhibits hypocotyl elongation at concentrations that promote leaf and cotyledon expansion in the dark-grown seedlings.

It is likely that the inhibition of hypocotyl and internode elongation induced by excess cytokinin is due to the production of ethylene, and this inhibition thus may represent another example of the interdependence of hormonal regulatory pathways.

On the other hand, other experiments suggest that endogenous cytokinins at normal physiological concentrations inhibit root growth.

Cytokinin-Regulated Processes Are Revealed in Plants That Overproduce Cytokinin

The *ipt* gene from the *Agrobacterium* Ti plasmid has been introduced into many species of plants, resulting in cytokinin overproduction. These transgenic plants exhibit an array of developmental abnormalities that tell us a great deal about the biological role of cytokinins.

As discussed earlier, plant tissues transformed by *Agrobacterium* carrying a wild-type Ti plasmid proliferate as tumors as a result of the overproduction of both auxin and cytokinin. And as mentioned already, if all of the other genes in the T-DNA are deleted and plant tissues are transformed with T-DNA containing only a selective antibiotic resistance marker gene and the *ipt* gene, shoots proliferate instead of callus.
The shoot teratomas formed by *ipt*-transformed tissues are difficult to root, and when roots are formed, they tend to be stunted in their growth. As a result, it is difficult to obtain plants from shoots expressing the *ipt* gene under the control of its own promoter because the promoter is a constitutive promoter and the gene is continuously expressed. To circumvent this problem, a variety of promoters whose

expression can be regulated have been used to drive the expression of the *ipt* gene in the transformed tissues.

For example, several studies have employed a heat shock promoter, which is induced in response to elevated temperature, to drive inducible expression of the *ipt* gene in transgenic tobacco and *Arabidopsis*. In these plants, heat induction substantially increased the level of zeatin, zeatin riboside and ribotide, and *N*-conjugated zeatin.

These cytokinin-overproducing plants exhibit several characteristics that point to roles played by cytokinin in plant physiology and development:

- The shoot apical meristems of cytokinin-overproducing plants produce more leaves.
- The leaves have higher chlorophyll levels and are much greener.
- Adventitious shoots may form from unwounded leaf veins and petioles.
- Leaf senescence is retarded.
- Apical dominance is greatly reduced.
- The more extreme cytokinin-overproducing plants are stunted, with greatly shortened internodes.
- Rooting of stem cuttings is reduced, as is the root growth rate.

Some of the consequences of cytokinin overproduction could be highly beneficial for agriculture if synthesis of the hormone can be controlled. Because leaf senescence is delayed in the cytokinin-overproducing plants, it should be possible to extend their photosynthetic productivity.

In addition, cytokinin production could be linked to damage caused by predators. For example, tobacco plants transformed with an *ipt* gene under the control of the promoter from a wound-inducible protease inhibitor II gene were more resistant to insect damage. The tobacco hornworm consumed up to 70% fewer tobacco leaves in plants that expressed the *ipt* gene driven by the protease inhibit or promoter.

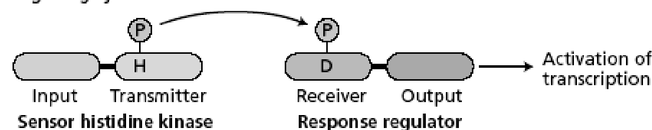
CELLULAR AND MOLECULAR MODES OF CYTOKININ ACTION

The diversity of the effects of cytokinin on plant growth and development is consistent with the involvement of signal transduction pathways with branches leading to specific responses. Although our knowledge of how cytokinin works at the cellular and molecular levels is still quite fragmentary, significant progress has been achieved. In this section we will discuss the nature of the cytokinin receptor and various cytokinin-regulated genes, as well as a model for cytokinin signaling based on current information.

A Cytokinin Receptor Related to Bacterial Two-Component Receptors Has Been Identified

The first clue to the nature of the cytokinin receptor came from the discovery of the *CKI1* gene. *CKI1* was identified in a screen for genes that, when overexpressed, conferred cytokinin-independent growth on *Arabidopsis* cells in culture.
As discussed already, plant cells generally require cytokinin in order to divide in culture. However, a cell line that overexpresses *CKI1* is capable of growing in culture in the absence of added cytokinin.

CKI1 encodes a protein similar in sequence to bacterial two-component sensor histidine kinases, which are ubiquitous receptors in prokaryotes. Bacterial two-component regulatory systems mediate a range of responses to environmental stimuli, such as osmoregulation and chemotaxis. Typically these systems are composed of two functional elements: a *sensor histidine kinase*, to which a signal binds, and a downstream *response regulator*, whose activity is regulated via phosphorylation by the sensor histidine kinase. The sensor histidine kinase is usually a membrane-bound protein that contains two distinct domains, called the input and histidine kinase, or “transmitter,” domains (Figure).



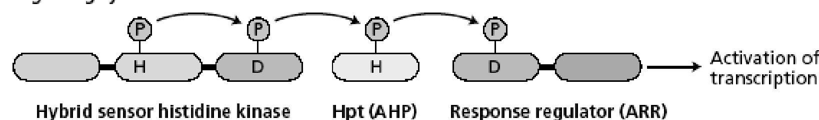


FIGURE : Simple versus phosphorelay types of two-component signaling systems. (A) In simple two-component systems, the input domain is the site where the signal is sensed. This regulates the activity of the histidine kinase domain, which when activated autophosphorylates on a conserved His residue. The phosphate is then transferred to an Asp residue that resides within the receiver domain of a response regulator. Phosphorylation of this Asp regulates the activity of the output domain of the response regulator, which in many cases is a transcription factor. (B) In the phosphorelay-type two-component signaling system, an extra set of phosphotransfers is mediated by a histidine phosphotransfer protein (Hpt), called AHP in *Arabidopsis*. The *Arabidopsis* response regulators are called ARRs. H = histidine, D = aspartate.

Detection of a signal by the input domain alters the activity of the histidine kinase domain. Active sensor kinases are dimers that transphosphorylate a conserved histidine residue. This phosphate is then transferred to a conserved aspartate residue in the receiver domain of a cognate response regulator, and this phosphorylation alters the activity of the kinases. Most response regulators also contain *output* domains that act as transcription factors.

The phenotype resulting from *CKI1* overexpression, combined with its similarity to bacterial receptors, suggested that the CKI1 and/or similar histidine kinases are cytokinin receptors. Support for this model came from identification of the *CRE1* gene.

Like *CKI1*, *CRE1* encodes a protein similar to bacterial histidine kinases. Loss-of-function *cre1* mutations were identified in a genetic screen for mutants that failed to develop shoots from undifferentiated tissue culture cells in response to cytokinin. This is essentially the opposite screen from the one just described, from which the *CKI1* gene was identified by a gain-of-function (ability to divide in the absence of cytokinin) mutation. The *cre1* mutants are also resistant to the inhibition of root elongation observed in response to cytokinin.

Convincing evidence that *CRE1* encodes a cytokinin receptor came from analysis of the expression of the protein in yeast. Yeast cells also contain a sensor histidine kinase, and deletion of the gene that encodes this kinase— *SLN1*—is lethal. Expression of *CRE1* in *SLN1*-deficient yeast can restore viability, *but only if cytokinins are present in the medium*. Thus the activity of CRE1 (i.e., its ability to replace SLN1) is dependent on cytokinin, which, coupled with the cytokinin-insensitive phenotype of the *cre1* mutants in *Arabidopsis*, unequivocally demonstrates that CRE1 is a cytokinin receptor. It remains to be determined if CKI1 is also a cytokinin receptor.

Two other genes in the *Arabidopsis* genome (*AHK2* and *AHK3*) are closely related to *CRE1*, suggesting that, like the ethylene receptors, the cytokinin receptors are encoded by a multigene family. Indeed, it has been demonstrated that cytokinins bind to the predicted extracellular domains of CRE1, AHK2, and AHK3 with high affinity, confirming that they are indeed cytokinin receptors. This raises the possibility that these genes are at least partially genetically redundant (as are the ethylene receptors), which may explain the relatively mild phenotypes that result from loss-of-function *cre1* mutations.

Cytokinins Cause a Rapid Increase in the Expression of Response Regulator Genes

One of the primary effects of cytokinin is to alter the expression of various genes. The first set of genes to be upregulated in response to cytokinin are the ***ARR*** (*A*rabidopsis *r*esponse *r*egulator) genes. These genes are homologous to the receiver domain of bacterial two-component response regulators, the downstream target of sensor histidine kinases.

In *Arabidopsis*, response regulators are encoded by a multigene family. They fall into two basic classes: the **type-A *ARR*** genes, which are made up solely of a receiver domain, and the **type-B *ARR*** genes, which contain a transcription factor domain in addition to the receiver domain. The rate of transcription of the type-A gene is increased within 10 minutes in response to applied cytokinin. This rapid induction is specific for cytokinin and does not require new protein synthesis. Both of these features are hallmarks of primary response genes.

The rapid induction of the type-A genes, coupled with their similarity to signaling elements predicted to act downstream of sensor histidine kinases, suggests that these elements act downstream of the CRE1 cytokinin receptor family to mediate the primary cytokinin response. Interestingly, one of these type-A genes, *ARR5*, is expressed primarily in the apical meristems of both shoots and roots, consistent with a role in regulating cell proliferation, a key aspect of cytokinin action.

The expression of a wide variety of other genes is altered in response to cytokinin, but generally with slower kinetics than the type-A genes. These include the gene that encodes nitrate reductase, light-regulated genes such as *LHCB* and *SSU*, and defense-related genes such as *PR1*, as well as genes that encode an extensin (cell wall protein rich in hydroxyproline), rRNAs, cytochrome P450s, and peroxidase. Cytokinin elevates the expression of these genes both by increasing the rate of transcription (as in the case of the type-A *ARR*s) and/or by a stabilization of the RNA transcript (e.g., the extensin gene).

Histidine Phosphotransferases May Mediate the Cytokinin Signaling Cascade

From the preceding discussions we have seen that cytokinin binds to the CRE1 receptors to initiate a response that culminates in the elevation of transcription of the type- A *ARR*s. The type-A ARR proteins, in turn, may regulate the expression of numerous other genes, as well as the activities of various target proteins that ultimately alter cellular function. How is the signal propagated from CRE1 (which is at the plasma membrane) to the nucleus to alter type-A *ARR* transcription?

One set of genes that are likely to be involved in this signaling cascade encode the **AHP** (*A*rabidopsis *h*istidine *p*hosphotransfer) proteins. In two-component systems that involve a sensor kinase fused to a receiver domain (the structure of most eukaryotic sensor histidine kinases, including those of the CRE1 family), there is an additional set of phosphotransfers that are mediated by a **histidine phosphotransfer protein** (**Hpt**).

Phosphate is first transferred from ATP to a histidine within the histidine kinase domain, and then transferred to an aspartate residue on the fused receiver. From the aspartate residue the phosphate group is then transferred to a histidine on the Hpt protein and then finally to an aspartate on the receiver domain of the response regulator. This phosphorylation of the receiver domain of the response regulator alters its activity. Thus, Hpt proteins are predicted to mediate the phosphotransfer between sensor kinases and response regulators.

In *Arabidopsis* there are 5 Hpt genes, called *AHP*s. The AHP proteins have been shown to physically associate with receiver domains from several *Arabidopsis* histidine kinases, including CRE1, and a subset of the AHPs have been demonstrated to transiently translocate from the cytoplasm to the nucleus in response to cytokinin. This finding suggests that the AHPs are the immediate downstream targets of the activated CRE receptors, and that these proteins transduce the cytokinin signal into the nucleus.

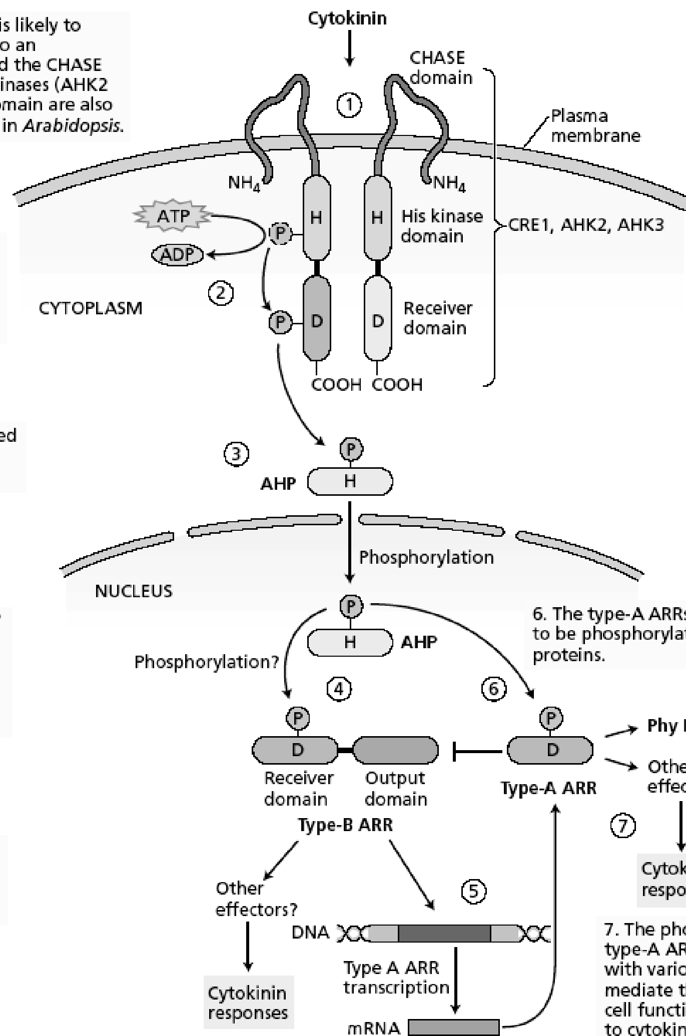
1. Cytokinin binds to CRE1, which is likely to occur as a dimer. Cytokinin binds to an extracellular portion of CRE1 called the CHASE domain.Two other hybrid sensor kinases (AHK2 and AHK3) containing a CHASE domain are also likely to act as cytokinin receptors in *Arabidopsis*.

2. Cytokinin binding to these receptors activates their histidine kinase activity. The phosphate is transferred to an asparate residue (D) on the fused receiver domains.

3. The phosphate is then transferred to a conserved histidine present in an AHP protein.

4. Phosphorylation causes the AHP protein to move into the nucleus, where it transfers the phosphate to an asparate residue located within the receiver domain of a type-B ARR.

5. The phosphorylation of the type-B ARR activates the output domain to induce transcription of genes encoding type-A ARRs.



6. The type-A ARRs are likely also to be phosphorylated by the AHP proteins.

7. The phosphorylated type-A ARRs interact with various effectors to mediate the changes in cell function appropriate to cytokinin (indicated in the model as "cytokinin responses").

FIGURE Model of cytokinin signaling. The near future should see significant refinement of this model, the tools are now in hand to analyze the interactions among these elements.

Cytokinin-Induced Phosphorylation Activates Transcription Factors

The question now becomes, How do the activated AHPs, once in the nucleus, act to regulate gene transcription? Genetic studies in intact *Arabidopsis* plants and overexpression studies in isolated *Arabidopsis* protoplasts using a cytokinin responsive reporter have provided a likely answer.

Disruption of *ARR1*, one of the type-B *ARR* genes, reduces the induction of the type-A*ARR* genes in response to cytokinin. Conversely, an increase in *ARR*1 function increases the response of the type-A genes to cytokinin. This suggests that ARR1, which is a transcription factor, directly regulates transcription of the type-A *ARR*s, and that by analogy other members of the type-B *ARR* family also mediate cytokinin-regulated gene expression.

This conclusion is supported by the findings that type- B ARRs operate as transcriptional activators and that there are multiple binding sites for ARR1, a type-B ARR, in the 5′ DNAregulatory sequences of the type-A ARR genes. A model of cytokinin signaling is presented in Figure. Cytokinin binds to the CRE1 receptor and initiates a phosphorylation cascade that results in the phosphorylation and activation of a subset of the type-B ARR proteins. Activation of the type-B proteins (transcription factors) leads to the transcriptional activation of the type-A genes. The type-AARR proteins are likely also phosphorylated in response to cytokinin, and perhaps together with the type- B proteins, they interact with various targets to mediate the changes in cellular function, such as an activation of the cell cycle. Type-A ARRs are also able to inhibit their own expression by an unknown mechanism, providing a negative feedback loop (see Figure). Much work needs to be done to confirm and refine this model, but we are beginning to glimpse for the first time the molecular basis for cytokinin action in plants.

3. GIBBERLIC ACID

For nearly 30 years after the discovery of auxin in 1927, and more than 20 years after its structural elucidation as indole-3-acetic acid, Western plant scientists tried to ascribe the regulation of all developmental phenomena in plants to auxin. However, plant growth and development are regulated by several different types of hormones acting individually and in concert.

In the 1950s the second group of hormones, the gibberellins (GAs), was characterized. The gibberellins are a large group of related compounds (more than 125 are known) that, unlike the auxins, are defined by their chemical structure rather than by their biological activity. Gibberellins are most often associated with the promotion of stem growth, and the application of gibberellin to intact plants can induce large increases in plant height. As we will see, however, gibberellins play important roles in a variety of physiological phenomena.

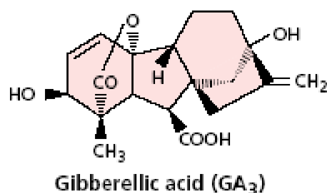
The biosynthesis of gibberellins is under strict genetic, developmental, and environmental control, and numerous gibberellin-deficient mutants have been isolated. Mendel's tall/dwarf alleles in peas are a famous example. Such mutants have been useful in elucidating the complex pathways of gibberellin biosynthesis.

THE DISCOVERY OF THE GIBBERELLINS

Although gibberellins did not become known to American and British scientists until the 1950s, they had been discovered much earlier by Japanese scientists. Rice farmers in Asia had long known of a disease that makes the rice plants grow tall but eliminates seed production. In Japan this disease was called the "foolish seedling," or *bakanae*, disease.

Plant pathologists investigating the disease found that the tallness of these plants was induced by a chemical secreted by a fungus that had infected the tall plants. This chemical was isolated from filtrates of the cultured fungus and called *gibberellin* after *Gibberella fujikuroi*, the name of the fungus.

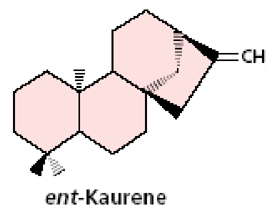
In the 1930s Japanese scientists succeeded in obtaining impure crystals of two fungal growth-active compounds, which they termed *gibberellin A* and *B*, but because of communication barriers and World War II, the information did not reach the West. Not until the mid-1950s did two groups—one at the Imperial Chemical Industries (ICI) research station at Welyn in Britain, the other at the U.S. Department of Agriculture (USDA) in Peoria, Illinois—succeed in elucidating the structure of the material that they had purified from fungal culture filtrates, which they named *gibberellic acid*:



Gibberellic acid (GA_3)

At about the same time scientists at Tokyo University isolated three gibberellins from the original gibberellin A and named them gibberellin A1, gibberellin A2, and gibberellin A3. Gibberellin A3 and gibberellic acid proved to be identical.

It became evident that an entire family of gibberellins exists and that in each fungal culture different gibberellins predominate, though gibberellic acid is always a principal component. As we will see, the structural feature that all gibberellins have in common, and that defines them as a family of molecules, is that they are derived from the *ent* kaurene ring structure:

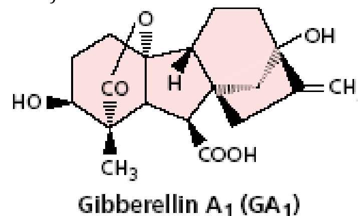


ent-Kaurene

As gibberellic acid became available, physiologists began testing it on a wide variety of plants. Spectacular responses were obtained in the elongation growth of dwarf and rosette plants, particularly in genetically dwarf peas (*Pisum sativum*), dwarf maize (*Zea mays*), and many rosette plants. In contrast, plants that were genetically very tall showed no further response to applied gibberellins. More recently, experiments with dwarf peas and dwarf corn have confirmed that the natural elongation growth of plants is regulated by gibberellins, as we will describe later.

Because applications of gibberellins could increase the height of dwarf plants, it was natural to ask whether plants contain their own gibberellins. Shortly after the discovery of the growth effects of gibberellic acid, gibberellin-like substances were isolated from several species of plants. *Gibberellin-like substance* refers to a compound or an extract that has gibberellin-like biological activity, but whose chemical structure has not yet been defined. Such a response indicates, but does not prove, that the tested substance is a gibberellin.

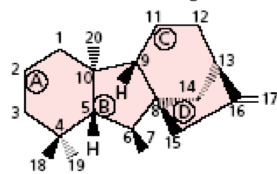
In 1958 a gibberellin (gibberellin A1) was conclusively identified from a higher plant (runner bean seeds, *Phaseolus coccineus*):



Gibberellin A_1 (GA_1)

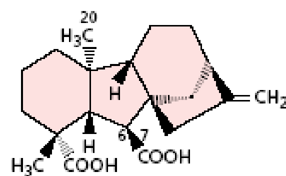
Because the concentration of gibberellins in immature seeds far exceeds that in vegetative tissue, immature seeds were the tissue of choice for gibberellin extraction. However, because the concentration of gibberellins in plants is very low (usually 1–10 parts per billion for the active gibberellin in vegetative tissue and up to 1 part per million of total gibberellins in seeds), chemists had to use truckloads of seeds. As more and more gibberellins from fungal and plant sources were characterized, they were numbered as gibberellin AX (or GAX), where *X* is a number, in the order of their discovery. This scheme was adopted for all gibberellins in 1968. However, the number of a gibberellin is simply a cataloging convenience, designed to prevent chaos in the naming of the gibberellins. The system implies no close chemical similarity or metabolic relationship between gibberellins with adjacent numbers.

All gibberellins are based on the *ent*-gibberellane skeleton:



ent-Gibberellane structure

Some gibberellins have the full complement of 20 carbons (C20-GAs):



GA_{12} (a C_{20}-gibberellin)

Others have only 19 (C19-GAs), having lost one carbon to metabolism. There are other variations in the basic structure, especially the oxidation state of carbon 20 (in C20-GAs) and the number and position of hydroxyl groups on the molecule. Despite the plethora of gibberellins present in plants, genetic analyses have demonstrated that only a few are biologically active as hormones. All the others serve as precursors or represent inactivated forms.

EFFECTS OF GIBBERELLIN ON GROWTH AND DEVELOPMENT

Though they were originally discovered as the cause of a disease of rice that stimulated internode elongation, endogenous gibberellins influence a wide variety of developmental processes. In addition to stem elongation, gibberellins control various aspects of seed germination, including the loss of dormancy and the mobilization of endosperm reserves. In reproductive development, gibberellin can affect the transition from the juvenile to the mature stage, as well as floral initiation, sex determination, and fruit set. In this section we will review some of these gibberellin-regulated phenomena.

Gibberellins Stimulate Stem Growth in Dwarf and Rosette Plants

Applied gibberellin promotes internodal elongation in a wide range of species. However, the most dramatic stimulations are seen in dwarf and rosette species, as well as members of the grass family. Exogenous GA3 causes such extreme stem elongation in dwarf plants that they resemble the tallest varieties of the same species.

Accompanying this effect are a decrease in stem thickness, a decrease in leaf size, and a pale green color of the leaves. Some plants assume a rosette form in short days and undergo shoot elongation and flowering only in long days. Gibberellin application results in *bolting* (stem growth) in plants kept in short days, and normal bolting is regulated by endogenous gibberellin. In addition, as noted earlier, many long-day rosette plants have a cold requirement for stem elongation and flowering, and this requirement is overcome by applied gibberellin. GA also promotes internodal elongation in members of the grass family. The target of gibberellin action is the **intercalary meristem**—a meristem near the base of the internode that produces derivatives above and below. Deep water rice is a particularly striking example.

Although stem growth may be dramatically enhanced by GAs, gibberellins have little direct effect on root growth. However, the root growth of extreme dwarfs is less than that of wild-type plants, and gibberellin application to the shoot enhances both shoot and root growth. Whether the effect of gibberellin on root growth is direct or indirect is currently unresolved.

Gibberellins Regulate the Transition from Juvenile to Adult Phases

Many woody perennials do not flower until they reach a certain stage of maturity; up to that stage they are said to be juvenile. The juvenile and mature stages often have different leaf forms, as in English ivy (*Hedera helix*). Applied gibberellins can regulate this juvenility in both directions, depending on the species. Thus, in English ivy GA3 can cause a reversion from a mature to a juvenile state, and many juvenile conifers can be induced to enter the reproductive phase by applications of non polar gibberellins such as GA4 + GA7. (The latter example is one instance in which GA3 is not effective.)

Gibberellins Influence Floral Initiation and Sex Determination

As already noted, gibberellin can substitute for the long day or cold requirement for flowering in many plants, especially rosette species. Gibberellin is thus a component of the flowering stimulus in some plants, but apparently not in others.

In plants where flowers are unisexual rather than hermaphroditic, floral sex determination is genetically regulated. However, it is also influenced by environmental factors, such as photoperiod and nutritional status, and these environmental effects may be mediated by gibberellin. In maize, for example, the staminate flowers (male) are restricted to the tassel, and the pistillate flowers (female) are contained in the ear. Exposure to short days and cool nights increases the endogenous gibberellin levels in the tassels 100-fold and simultaneously causes feminization of the tassel flowers. Application of exogenous gibberellic acid to the tassels can also induce pistillate flowers. For studies on genetic regulation, a large collection of maize mutants that have altered patterns of sex determination have been isolated. Mutations in genes that affect either gibberellin biosynthesis or gibberellin signal transduction result in a failure to suppress stamen development in the flowers of the ear. Thus the primary role of gibberellin in sex determination in maize seems to be to suppress stamen development.

In dicots such as cucumber, hemp, and spinach, gibberellin seems to have the opposite effect. In these species, application of gibberellin promotes the formation of staminate flowers, and inhibitors of gibberellin biosynthesis promote the formation of pistillate flowers.

Gibberellins Promote Fruit Set

Applications of gibberellins can cause *fruit set* (the initiation of fruit growth following pollination) and growth of some fruits, in cases where auxin may have no effect. For example, stimulation of fruit set by gibberellin has been observed in apple (*Malus sylvestris*).

Gibberellins Promote Seed Germination

Seed germination may require gibberellins for one of several possible steps: the activation of vegetative growth of the embryo, the weakening of a growth-constraining endosperm layer surrounding the embryo, and the mobilization of stored food reserves of the endosperm. Some seeds, particularly those of wild plants, require light or cold to induce germination. In such seeds this dormancy can often be overcome by application of gibberellin.

Since changes in gibberellin levels are often, but not always, seen in response to chilling of seeds, gibberellins may represent a natural regulator of one or more of the processes involved in germination. Gibberellin application also stimulates the production of numerous hydrolases, notably α-amylase, by the aleurone layers of germinating cereal grains. This aspect of gibberellin action has led to its use in the brewing industry in the production of malt.

Gibberellins Have Commercial Applications

The major uses of gibberellins (GA3, unless noted otherwise), applied as a spray or dip, are to manage fruit crops, to malt barley, and to increase sugar yield in sugarcane. In some crops a reduction in height is desirable, and this can be accomplished by the use of gibberellin synthesis inhibitors.

Fruit production. A major use of gibberellins is to increase the stalk length of seedless grapes. Because of the shortness of the individual fruit stalks, bunches of seedless grapes are too compact and the growth of the berries is restricted. Gibberellin stimulates the stalks to grow longer, thereby allowing the grapes to grow larger by alleviating compaction, and it promotes elongation of the fruit.

A mixture of benzyladenine (a cytokinin) and GA4 + GA7 can cause apple fruit to elongate and is used to improve the shape of Delicious-type apples under certain conditions. Although this treatment does not affect yield or taste, it is considered commercially desirable. In citrus fruits, gibberellins delay senescence, allowing the fruits to be left on the tree longer to extend the market period.

Malting of barley. Malting is the first step in the brewing process. During malting, barley seeds (*Hordeum vulgare*) are allowed to germinate at temperatures that maximize the production of hydrolytic enzymes by the aleurone layer. Gibberellin is sometimes used to speed up the malting process. The germinated seeds are then dried and pulverized to produce "malt," consisting mainly of a mixture of amylolytic (starch-degrading) enzymes and partly digested starch.

During the subsequent "mashing" step, water is added and the amylases in the malt convert the residual starch, as well as added starch, to the disaccharide maltose, which is converted to glucose by the enzyme maltase. The resulting "wort" is then boiled to stop the reaction. In the final step, yeast converts the glucose in the wort to ethanol by fermentation.

Increasing sugarcane yields. Sugarcane (*Saccharum officinarum*) is one of relatively few plants that store their carbohydrate as sugar (sucrose) instead of starch (the other important sugar-storing crop is sugar beet). Originally from New Guinea, sugarcane is a giant perennial grass that can grow from 4 to 6 m tall. The sucrose is stored in the central vacuoles of the internode parenchyma cells. Spraying the crop with gibberellin can increase the yield of raw cane by up to 20 tons per acre, and the sugar yield by 2 tons per acre. This increase is a result of the stimulation of internode elongation during the winter season.

Uses in plant breeding. The long juvenility period in conifers can be detrimental to a breeding program by preventing the reproduction of desirable trees for many years. Spraying with GA4 + GA7 can considerably reduce the time to seed production by inducing cones to form on very young trees. In addition, the promotion of male flowers in cucurbits, and the stimulation of bolting in biennial rosette crops such as beet (*Beta vulgaris*) and cabbage (*Brassica oleracea*), are beneficial effects of gibberellins that are occasionally used commercially in seed production.

Gibberellin biosynthesis inhibitors. Bigger is not always better. Thus, gibberellin biosynthesis inhibitors are used commercially to prevent elongation growth in some plants. In floral crops, short, stocky plants such as lilies, chrysanthemums, and poinsettias are desirable, and restrictions on elongation growth can be achieved by applications of gibberellin synthesis inhibitors such as ancymidol (known commercially as A-Rest) or paclobutrazol (known as Bonzi). Tallness is also a disadvantage for cereal crops grown in cool, damp climates, as occur in Europe, where lodging can be a problem. *Lodging*—the bending of stems to the ground caused by the weight of water collecting on the ripened heads—makes it difficult to harvest the grain with a combine harvester. Shorter internodes reduce the tendency of the plants to lodge, increasing the yield of the crop. Even genetically dwarf wheats grown in Europe are sprayed with gibberellin biosynthesis inhibitors to further reduce stem length and lodging. Yet another application of gibberellin biosynthesis inhibitors is the restriction of growth in roadside shrub plantings.

BIOSYNTHESIS AND METABOLISM OF GIBBERELLIN

Gibberellins constitute a large family of diterpene acids and are synthesized by a branch of the **terpenoid pathway**. The elucidation of the gibberellin biosynthetic pathway would not have been possible without the development of sensitive methods of detection. As noted earlier, plants contain a bewildering array of gibberellins, many of which are *biologically inactive*. In this section we will discuss the biosynthesis of GAs, as well as other factors that regulate the steady-state levels of the biologically active form of the hormone in different plant tissues.

Gibberellins Are Measured via Highly Sensitive Physical Techniques

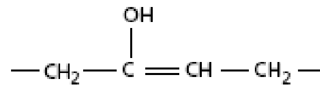
Systems of measurement using a biological response, called *bioassays*, were originally important for detecting gibberellin- like activity in partly purified extracts and for assessing the biological activity of known gibberellins. The use of bioassays, however, has declined with the development of highly sensitive physical techniques that allow precise identification and quantification of specific gibberellins from small amounts of tissue.

High-performance liquid chromatography (HPLC) of plant extracts, followed by the highly sensitive and selective analytical method of gas chromatography combined with mass spectrometry (GC-MS), has now become the method of choice. With the availability of published mass spectra, researchers can now identify gibberellins without possessing pure standards. The availability of heavy-isotope- labeled standards of common gibberellins, which

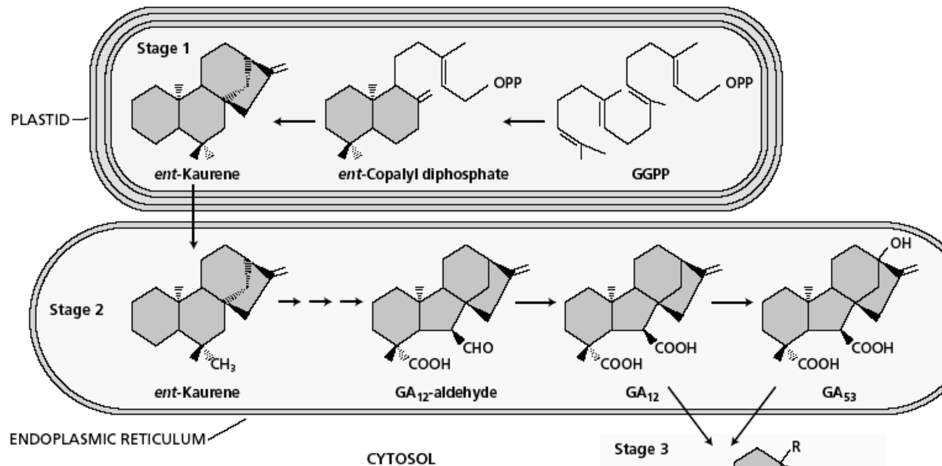
can themselves be separately detected on a mass spectrometer, allows the accurate measurement of levels in plant tissues by mass spectrometry with these heavy-isotope-labeled gibberellins as internal standards for quantification.

Gibberellins Are Synthesized via the Terpenoid Pathway in Three Stages

Gibberellins are tetracyclic diterpenoids made up of four isoprenoid units. Terpenoids are compounds made up of five-carbon (isoprene) building blocks:



joined head to tail. Researchers have determined the entire gibberellin biosynthetic pathway in seed and vegetative tissues of several species by feeding various radioactive precursors and intermediates and examining the production of the other compounds of the pathway. The gibberellin biosynthetic pathway can be divided into three stages, each residing in a different cellular compartment (Figure).



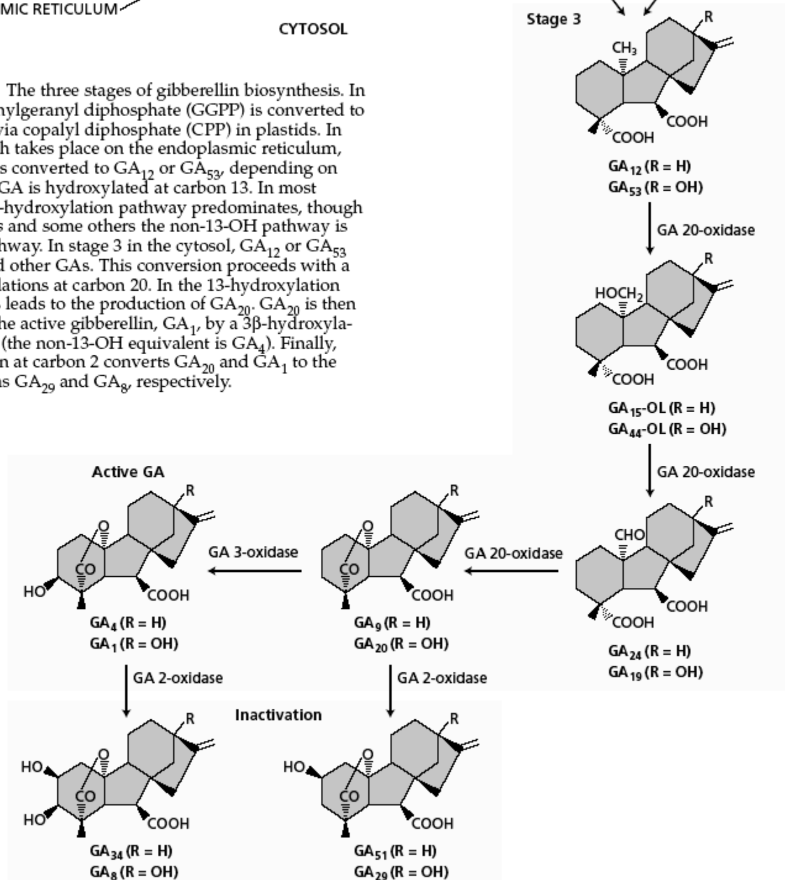


FIGURE The three stages of gibberellin biosynthesis. In stage 1, geranylgeranyl diphosphate (GGPP) is converted to *ent*-kaurene via copalyl diphosphate (CPP) in plastids. In stage 2, which takes place on the endoplasmic reticulum, *ent*-kaurene is converted to GA_{12} or GA_{53}, depending on whether the GA is hydroxylated at carbon 13. In most plants the 13-hydroxylation pathway predominates, though in *Arabidopsis* and some others the non-13-OH pathway is the main pathway. In stage 3 in the cytosol, GA_{12} or GA_{53} are converted other GAs. This conversion proceeds with a series of oxidations at carbon 20. In the 13-hydroxylation pathway this leads to the production of GA_{20}. GA_{20} is then oxidized to the active gibberellin, GA_1, by a 3β-hydroxylation reaction (the non-13-OH equivalent is GA_4). Finally, hydroxylation at carbon 2 converts GA_{20} and GA_1 to the inactive forms GA_{29} and GA_8, respectively.

Stage 1: Production of terpenoid precursors and ent-kaurene in plastids. The basic biological isoprene unit is isopentenyl diphosphate (IPP).2 IPP used in gibberellin biosynthesis in green tissues is synthesized in plastids from glyceraldehyde-3-phosphate and pyruvate. However, in the endosperm of pumpkin seeds, which are very rich in gibberellin, IPP is formed in the cytosol from mevalonic acid, which is itself derived from acetyl-CoA.

Thus the IPP used to make gibberellins may arise from different cellular compartments in different tissues. Once synthesized, the IPP isoprene units are added successively to produce intermediates of 10 carbons (geranyl diphosphate), 15 carbons (farnesyl diphosphate), and 20 carbons (geranylgeranyl diphosphate, GGPP). GGPP is a precursor of many terpenoid compounds, including carotenoids and many essential oils, and it is only after GGPP that the pathway becomes specific for gibberellins.

The cyclization reactions that convert GGPP to *ent*-kaurene represent the first step that is specific for the gibberellins. The two enzymes that catalyze the reactions are localized in the proplastids of meristematic shoot tissues, and they are not present in mature chloroplasts. Thus, leaves lose their ability to synthesize gibberellins from IPP once their chloroplasts mature.

Compounds such as AMO-1618, Cycocel, and Phosphon D are specific inhibitors of the first stage of gibberellin biosynthesis, and they are used as growth height reducers.

Stage 2: Oxidation reactions on the ER form GA12 and GA53. In the second stage of gibberellin biosynthesis, a methyl group on *ent*-kaurene is oxidized to a carboxylic acid, followed by contraction of the B ring from a six- to a five-carbon ring to give GA12-aldehyde. GA12-aldehyde is then oxidized to **GA12**, the first gibberellin in the pathway in all plants and thus the precursor of all the other gibberellins.

Many gibberellins in plants are also hydroxylated on carbon 13. The hydroxylation of carbon 13 occurs next, forming GA53 from GA12. All the enzymes involved are monooxygenases that utilize cytochrome P450 in their reactions.

These P450 monooxygenases are localized on the endoplasmic reticulum. Kaurene is transported from the plastid to the endoplasmic reticulum, and is oxidized *en route* to kaurenoic acid by kaurene oxidase, which is associated with the plastid envelope.

Further conversions to GA12 take place on the endoplasmic reticulum. Paclobutrazol and other inhibitors of P450 monooxygenases specifically inhibit this stage of gibberellin biosynthesis before GA12-aldehyde, and they are also growth retardants.

Stage 3: Formation in the cytosol of all other gibberellins from GA12 or GA53. All subsequent steps in the pathway are carried out by a group of soluble dioxygenases in the cytosol. These enzymes require 2- oxoglutarate and molecular oxygen as cosubstrates, and they use Fe2+ and ascorbate as cofactors. The specific steps in the modification of GA12 vary from species to species, and between organs of the same species.

Two basic chemical changes occur in most plants:
1. Hydroxylation at carbon 13 (on the endoplasmic reticulum) or carbon 3, or both.
2. A successive oxidation at carbon 20 ($CH_2 \rightarrow CH_2OH \rightarrow CHO$). The final step of this oxidation is the loss of carbon 20 as CO_2.

When these reactions involve gibberellins initially hydroxylated at C-13, the resulting gibberellin is GA20. GA20 is then converted to the biologically active form, GA1, by hydroxylation of carbon 3. (Because this is in the beta configuration [drawn as if the bond to the hydroxyl group were toward the viewer], it is referred to as 3β-hydroxylation.)

Finally, GA1 is inactivated by its conversion to GA8 by a hydroxylation on carbon 2. This hydroxylation can also remove GA20 from the biosynthetic pathway by converting it to GA29.

Inhibitors of the third stage of the gibberellin biosynthetic pathway interfere with enzymes that utilize 2-oxoglutarate as cosubstrates. Among these, the compound prohexadione (BX-112), is especially useful because it specifically inhibits GA 3-oxidase, the enzyme that converts inactive GA20 to growth-active GA1.

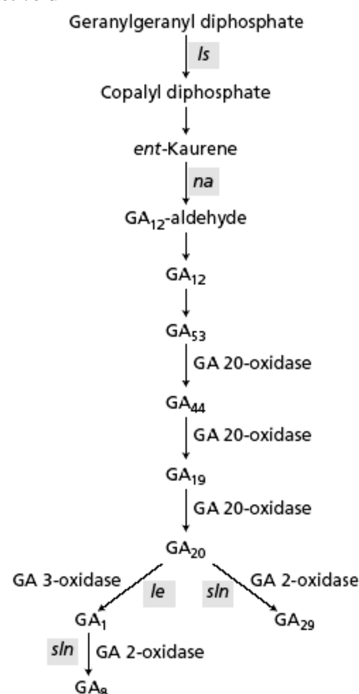


FIGURE A portion of the gibberellin biosynthetic pathway showing the abbreviations and location of the mutant genes that block the pathway in pea and the enzymes involved in the metabolic steps after GA_{53}.

The Enzymes and Genes of the Gibberellin Biosynthetic Pathway Have Been Characterized

The enzymes of the gibberellin biosynthetic pathway are now known, and the genes for many of these enzymes have been isolated and characterized. Most notable from a regulatory standpoint are two biosynthetic enzymes—GA 20-oxidase (GA20ox)3 and GA 3-oxidase (GA3ox)—and an enzyme involved in gibberellin metabolism, GA 2-oxidase (GA2ox):

• **GA 20-oxidase** catalyzes all the reactions involving the successive oxidation steps of carbon 20 between GA53 and GA20, including the removal of C-20 as CO2.
• **GA 3-oxidase** functions as a β-hydroxylase, adding a hydroxyl group to C-3 to form the active gibberellin, GA1. (The evidence demonstrating that GA1 is the active gibberellin will be discussed shortly.)
• **GA 2-oxidase** *inactivates* GA1 by catalyzing the addition of a hydroxyl group to C-2.

The transcription of the genes for the two gibberellin biosynthetic enzymes, as well as for GA 2-oxidase, is highly regulated. All three of these genes have sequences in common with each other and with other enzymes utilizing 2-oxoglutarate and Fe^{2+} as cofactors. The common sequences represent the binding sites for 2-oxoglutarate and Fe2+.

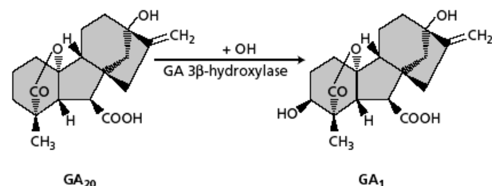
Gibberellins May Be Covalently Linked to Sugars

Although active gibberellins are free, a variety of gibberellin glycosides are formed by a covalent linkage between gibberellin and a sugar. These gibberellin conjugates are particularly prevalent in some seeds. The conjugating sugar is usually glucose, and it may be attached to the gibberellin via a carboxyl group forming a gibberellin glycoside, or via a hydroxyl group forming a gibberellin glycosyl ether.

When gibberellins are applied to a plant, a certain proportion usually becomes glycosylated. Glycosylation therefore represents another form of inactivation. In some cases, applied glucosides are metabolized back to free GAs, so glucosides may also be a storage form of gibberellins.

GA1 Is the Biologically Active Gibberellin Controlling Stem Growth

Knowledge of biosynthetic pathways for gibberellins reveals where and how dwarf mutations act. Although it had long been assumed that gibberellins were natural growth regulators because gibberellin application caused dwarf plants to grow tall, direct evidence was initially lacking. In the early 1980s it was demonstrated that tall stems do contain more bioactive gibberellin than dwarf stems have, and that the level of the endogenous bioactive gibberellin mediates the genetic control of tallness.



The gibberellins of tall pea plants containing the homozygous *Le* allele (wild type) were compared with dwarf plants having the same genetic makeup, except containing the *le* allele (mutant). *Le* and *le* are the two alleles of the gene that regulates tallness in peas, the genetic trait first investigated by Gregor Mendel in his pioneering study in 1866. We now know that tall peas contain much more bioactive GA1 than dwarf peas have (Ingram et al. 1983). As we have seen, the precursor of GA1 in higher plants is GA20 (GA1 is 3β-OH GA20). If GA20 is applied to homozygous dwarf (*le*) pea plants, they fail to respond, although they do respond to applied GA1. The implication is that the *Le* gene enables the plants to convert GA20 to GA1. Metabolic studies using both stable and radioactive isotopes demonstrated conclusively that the *Le* gene encodes an enzyme that 3β-hydroxylates GA20 to produce GA1 (Figure).

Mendel's *Le(T)* gene was isolated, and the recessive *le (t)* allele was shown to have a single base change leading to a defective enzyme only one-twentieth as active as the wild-type enzyme, so much less GA1 is produced and the plants are dwarf.

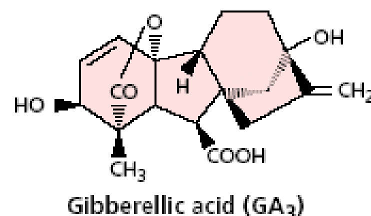
Endogenous GA1 Levels Are Correlated with Tallness

Although the shoots of gibberellin-deficient *le* dwarf peas are much shorter than those of normal plants (internodes of 3 cm in mature dwarf plants versus 15 cm in mature normal plants), the mutation is "leaky" (i.e., the mutated gene produces a partially active enzyme) and some endogenous GA1 remains to cause growth. Different *le* alleles give rise to peas differing in their height, and the height of the plant has been correlated with the amount of endogenous GA1.

There is also an extreme dwarf mutant of pea that has even fewer gibberellins. This dwarf has the allele *na* (the wild-type allele is *Na*), which completely blocks gibberellin biosynthesis between *ent*-kaurene and GA12-aldehyde. As a result, homozygous (*nana*) mutants, which are almost completely free of gibberellins, achieve a stature of only about 1 cm at maturity. However, *nana* plants may still possess an active GA 3β- hydroxylase encoded by *Le*, and thus can convert GA20 to GA1. If a *nana naLe* shoot is grafted onto a dwarf *le* plant, the resulting plant is tall because the *nana* shoot tip can convert the GA20 from the dwarf into GA1.

Such observations have led to the conclusion that GA1 is the biologically active gibberellin that regulates tallness in peas. The same result has been obtained for maize, a monocot, in parallel studies using genotypes that have blocks in the gibberellin biosynthetic pathway. Thus the control of stem elongation by GA1 appears to be universal.

Although GA1 appears to be the primary active gibberellin in stem growth for most species, a few other gibberellins have biological activity in other species or tissues. For example, GA3, which differs from GA1 only in having one double bond, is relatively rare in higher plants but is able to substitute for GA1 in most bioassays:



GA4, which lacks an OH group at C-13, is present in both *Arabidopsis* and members of the squash family (Cucurbitaceae). It is as active as GA1, or even more active, in some bioassays, indicating that GA4 is a bioactive gibberellin in the species where it occurs. The structure of GA4 looks like this:

Gibberellin A_4 (GA_4)

Gibberellins Are Biosynthesized in Apical Tissues

The highest levels of gibberellins are found in immature seeds and developing fruits. However, because the gibberellin level normally decreases to zero in mature seeds, there is no evidence that seedlings obtain any active gibberellins from their seeds.

Work with pea seedlings indicates that the gibberellin biosynthetic enzymes and GA3ox are specifically localized in young, actively growing buds, leaves, and upper internodes. In *Arabidopsis*, GA20ox is expressed primarily in the apical bud and young leaves, which thus appear to be the principal sites of gibberellin synthesis.

The gibberellins that are synthesized in the shoot can be transported to the rest of the plant via the phloem. Intermediates of gibberellin biosynthesis may also be translocated in the phloem. Indeed, the initial steps of gibberellin biosynthesis may occur in one tissue, and metabolism to active gibberellins in another.

Gibberellins also have been identified in root exudates and root extracts, suggesting that roots can also synthesize gibberellins and transport them to the shoot via the xylem.

Gibberellin Regulates Its Own Metabolism

Endogenous gibberellin regulates its own metabolism by either switching on or inhibiting the transcription of the genes that encode enzymes of gibberellin biosynthesis and degradation (feedback and feed-forward regulation, respectively). In this way the level of active gibberellins is kept within a narrow range, provided that precursors are available and the enzymes of gibberellin biosynthesis and degradation are functional.

For example, the application of gibberellin causes a down-regulation of the biosynthetic genes—GA20ox and GA3ox—and an elevation in transcription of the degradative gene—GA2ox. A mutation in the GA 2-oxidase gene, which prevents GA1 from being degraded, is functionally equivalent to applying exogenous gibberellin to the plant, and produces the same effect on the biosynthetic gene transcription. Conversely, a mutation that lowers the level of active gibberellin, such as GA1, in the plant stimulates the transcription of the biosynthetic genes—GA20ox and GA3ox— and down-regulates the degradative enzyme—GA2ox. In peas this is particularly evident in very dwarf plants, such as those with a mutation in the *LS* gene (CPP synthase) or even more severely dwarf *na* plants (defective GA12-aldehyde synthase).

Environmental Conditions Can Alter the Transcription of Gibberellin Biosynthesis Genes

Gibberellins play an important role in mediating the effects of environmental stimuli on plant development. Environmental factors such as photoperiod and temperature can alter the levels of active gibberellins by affecting gene transcription for specific steps in the biosynthetic pathway.

Light regulation of GA1 biosynthesis. The presence of light has many profound effects. Some seeds germinate only in the light, and in such cases gibberellin application can stimulate germination in darkness. The promotion of germination by light has been shown to be due to increases in GA1 levels resulting from a light-induced increase in the transcription of the gene for GA3ox, which converts GA20 to GA1. This effect shows red/far-red photoreversibility and is mediated by phytochrome.

When a seedling becomes exposed to light as it emerges from the soil, it changes its form—a process referred to as de-etiolation. One of the most striking changes is a decrease in the rate of stem elongation such that the stem in the light is shorter than the one in the dark. Initially it was assumed that the light-grown plants would contain less GA1 than dark-grown plants. However, light-grown plants turned out to contain *more* GA1 than dark-grown plants—indicating that de-etiolation is a complex process involving changes in the level of GA1, as well as changes in the responsiveness of the plant to GA1. In peas, for example, the level of GA1 initially falls within 4 hours of exposure to light because of an increase in transcription of the gene for GA2ox, leading to an increase in GA1 breakdown. The level of GA1 remains low for a day but then increases, so that by 5 days there is a fivefold increase in the GA1 content of the stems, even though the stem elongation rate is lower. The reason that growth slows down despite the increase in GA1 level is that the plants are now severalfold *less sensitive* to the GA1 present. As will be discussed later in the chapter, sensitivity to active gibberellin is governed by components of the gibberellin signal transduction pathway.

Photoperiod regulation of GA1 biosynthesis. When plants that require long days to flower are shifted from short days to long days, gibberellin metabolism is altered. In spinach (*Spinacia oleracea*), in short days, when the plants maintain a rosette form, the level of gibberellins hydroxylated at carbon 13 is relatively low. In response to increasing day length, the shoots of spinach plants begin to elongate after about 14 long days. The levels of all the gibberellins of the carbon 13–hydroxylated gibberellin pathway (GA53 → GA44 → GA19→GA20→GA1→GA8) start to increase after about 4 days. Although the level of GA20 increases 16-fold during the first 12 days, it is the fivefold increase in GA1 that induces stem growth.

The dependence of stem growth on GA1 has been shown through the use of different inhibitors of gibberellin synthesis and metabolism. The inhibitors AMO-1618 and BX-112 both prevent internode elongation (bolting). The effect of AMO-1618, which blocks gibberellin biosynthesis prior to GA12-aldehyde, can be overcome by applications of GA20. However, the effect of another inhibitor, BX-112, which blocks the production of GA1 from GA20, can be overcome only by GA1. This result demonstrates that the rise in GA1 is the crucial factor in regulating spinach stem growth.

The level of GA20-oxidase mRNA in spinach tissues, which occurs in the highest amount in shoot tips and elongating stems, is increased under long-day conditions. The fact that GA 20- oxidase is the enzyme that converts GA53 to GA20 explains why the concentration of GA20 was found to be higher in spinach under long-day conditions.

Photoperiod control of tuber formation. Potato tuberization is another process regulated by photoperiod. Tubers form on wild potatoes only in short days (although the requirement for short days has been bred out of many cultivated varieties), and this tuberization can be blocked by applications of gibberellin. The transcription of GA20ox was found to fluctuate during the light–dark cycle, leading to lower levels of GA1 in short days. Potato plants overexpressing the GA20ox gene showed delayed tuberization, whereas trans-formation with the antisense gene for GA20ox promoted tuberization, demonstrating the importance of the transcription of this gene in the regulation of potato tuberization.

In general, de-etiolation, light-dependent seed germination, and the photoperiodic control of stem growth in rosette plants and tuberization in potato are all mediated by phytochromes. There is mounting evidence that many phytochrome effects are in part due to modulation of the levels of gibberellins through changes in the transcription of the genes for gibberellin biosynthesis and degradation.

Temperature effects. Cold temperatures are required for the germination of certain seeds (stratification) and for flowering in certain species (vernalization). For example, a prolonged cold treatment is required for both the stem elongation and the flowering of *Thlaspi arvense* (field pennycress), and gibberellins can substitute for the cold treatment.

In the absence of the cold treatment, *ent*-kaurenoic acid accumulates to high levels in the shoot tip, which is also the site of perception of the cold stimulus. After cold treatment and a return to high temperatures, the *ent*-kaurenoic acid is converted to GA9, the most active gibberellin for stimulating the flowering response. These results are consistent with a cold-induced increase in the activity of *ent*-kaurenoic acid hydroxylase in the shoot tip.

Auxin Promotes Gibberellin Biosynthesis

Although we often discuss the action of hormones as if they act singly, the net growth and development of the plant are the results of many combined signals. In addition, hormones can influence each other's biosynthesis so that the effects produced by one hormone may in fact be mediated by others.

For example, it has long been known that auxin induces ethylene biosynthesis. It is now evident that gibberellin can induce auxin biosynthesis and that auxin can induce gibberellin biosynthesis. If pea plants are decapitated, leading to a cessation in stem elongation, not only is the level of auxin lowered because its source has been removed, but the level of GA1 in the upper stem drops sharply. This change can be shown to be an auxin effect because replacing the bud with a supply of auxin restores the GA1 level.

The presence of auxin has been shown to promote the transcription of *GA3ox* and to repress the transcription of *GA2ox*. In the absence of auxin the reverse occurs. Thus the apical bud promotes growth not only through the direct biosynthesis of auxin, but also through the auxin-induced biosynthesis of GA1.

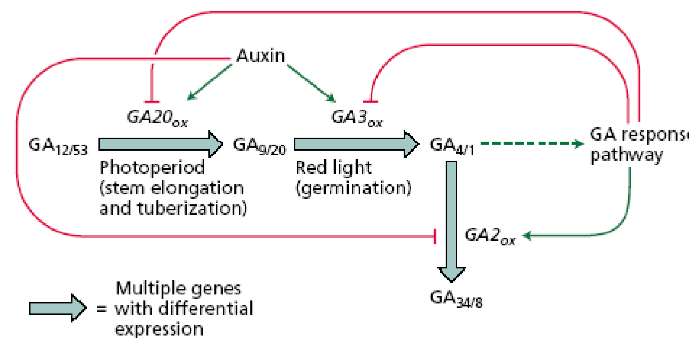


FIGURE The pathway of gibberellin biosynthesis showing the identities of the genes for the metabolic enzymes and the way that their transcription is regulated by feedback, environment, and other endogenous hormones.

Figure summarizes some of the factors that modulate the active gibberellin level through regulation of the transcription of the genes for gibberellin biosynthesis or metabolism.

Dwarfness Can Now Be Genetically Engineered

The characterization of the gibberellin biosynthesis and metabolism genes—*GA20ox*, *GA3ox*, and *GA2ox*—has enabled genetic engineers to modify the transcription of these genes to alter the gibberellin level in plants, and thus affect their height. The desired effect is usually to increase dwarfness because plants grown in dense crop communities, such as cereals, often grow too tall and thus are prone to lodging. In addition, because gibberellin regulates bolting, one can prevent bolting by inhibiting the rise in gibberellin. An example of the latter is the inhibition of bolting in sugar beet.

Sugar beet is a biennial, forming a swollen storage root in the first season and a flower and seed stalk in the second. To extend the growing season and obtain bigger beets, farmers sow the beets as early as possible in the spring, but sowing too early leads to bolting in the first year, with the result that no storage roots form. Areduction in the capacity to make gibberellin inhibits bolting, allowing earlier sowing of the seeds and thus the growth of larger beets. Reductions in GA1 levels have recently been achieved in such crops as sugar beet and wheat, either by the transformation of plants with antisense constructs of the *GA20ox* or *GA3ox* genes, which encode the enzymes leading to the synthesis of GA1, or by overexpressing the gene responsible for GA1 metabolism: *GA2ox*. Either approach results in dwarfing in wheat or an inhibition of bolting in rosette plants such as beet.

The inhibition of seed production in such transgenic plants can be overcome by sprays of gibberellin solution, provided that the reduction in gibberellin has been achieved by blocking the genes for GA20ox or GA3ox, the gibberellin biosynthetic enzymes. A similar strategy has recently been applied to turf grass, keeping the grass short with no seedheads, so that mowing can be virtually eliminated—a boon for homeowners!

PHYSIOLOGICAL MECHANISMS OF GIBBERELLIN-INDUCED GROWTH

As we have seen, the growth-promoting effects of gibberellin are most evident in dwarf and rosette plants. When dwarf plants are treated with gibberellin, they resemble the tallest varieties of the same species. Other examples of gibberellin action include the elongation of hypocotyls and of grass internodes.

A particularly striking example of internode elongation is found in deep-water rice (*Oryza sativa*). In general, rice plants are adapted to conditions of partial submergence. To enable the upper foliage of the plant to stay above water, the internodes elongate as the water level rises. Deep-water rice has the greatest potential for rapid internode elongation. Under field conditions, growth rates of up to 25 cm per day have been measured.

The initial signal is the reduced partial pressure of O_2 resulting from submergence, which induces ethylene biosynthesis. The ethylene trapped in the submerged tissues, in turn, reduces the level of abscisic acid, which acts as an antagonist of gibberellin. The end result is that the tissue becomes more responsive to its endogenous gibberellin. Because inhibitors of gibberellin biosynthesis block the stimulatory effect of both submergence and ethylene on growth, and exogenous gibberellin can stimulate growth in the absence of submergence, gibberellin appears to be the hormone directly responsible for growth stimulation.

GA-stimulated growth in deep-water rice can be studied in an excised stem system. The addition of gibberellin causes a marked increase in the growth rate after a lag period of about 40 minutes. Cell elongation accounts for about 90% of the length increase during the first 2 hours of gibberellin treatment.

Gibberellins Stimulate Cell Elongation and Cell Division

The effect of gibberellins applied to intact dwarf plants is so dramatic that it would seem to be a simple task to determine how they act. Unfortunately, this is not the case because, as we have seen with auxin, so much about plant cell growth is not understood. However, we do know some characteristics of gibberellin-induced stem elongation. Gibberellin increases both cell elongation and cell division, as evidenced by increases in cell length and cell number in response to applications of gibberellin. For example, internodes of tall peas have more cells than those of dwarf peas, and the cells are longer. Mitosis increases markedly in the subapical region of the meristem of rosette long-day plants after treatment with gibberellin. The dramatic stimulation of internode elongation in deep-water rice is due in part to increased cell division activity in the intercalary meristem. Moreover, only the cells of the intercalary meristem whose division is increased by gibberellin exhibit gibberellin-stimulated cell elongation.

Because gibberellin-induced cell elongation appears to precede gibberellin-induced cell division, we begin our discussion with the role of gibberellin in regulating cell elongation.

Gibberellins Enhance Cell Wall Extensibility without Acidification

The elongation rate can be influenced by both cell wall extensibility and the osmotically driven rate of water uptake. Gibberellin has no effect on the osmotic parameters but has consistently been observed to cause an increase in both the mechanical extensibility of cell walls and the stress relaxation of the walls of living cells. An analysis of pea genotypes differing in gibberellin content or sensitivity showed that gibberellin decreases the minimum force that will cause wall extension (the wall yield threshold). Thus, both gibberellin and auxin seem to exert their effects by modifying cell wall properties.

In the case of auxin, cell wall loosening appears to be mediated in part by cell wall acidification. However, this does not appear to be the mechanism of gibberellin action. In no case has a gibberellin-stimulated increase in proton extrusion been demonstrated. On the other hand, gibberellin is never present in tissues in the complete absence of auxin, and the effects of gibberellin on growth may depend on auxin-induced wall acidification. The typical lag time before gibberellin-stimulated growth begins is longer than for auxin; as noted already, in deepwater rice it is about 40 minutes, and in peas it is 2 to 3 hours. These longer lag times point to a growth-promoting mechanism distinct from that of auxin. Consistent with the existence of a separate gibberellin-
specific wall-loosening mechanism, the growth responses to applied gibberellin and auxin are additive. Various suggestions have been made regarding the mechanism of gibberellin-stimulated stem elongation, and all have some experimental support, but as yet none provide a clear-cut answer. For example, there is evidence that the enzyme xyloglucan endotransglycosylase (XET) is involved in gibberellin-promoted wall extension. The function of XET may be to facilitate the penetration of expansins into the cell wall. (Recall that expansins are cell wall proteins that cause wall loosening in acidic conditions by weakening hydrogen bonds between wall polysaccharides) Both expansins and XET may be required for gibberellin-stimulated cell elongation.

Gibberellins Regulate the Transcription of Cell Cycle Kinases in Intercalary Meristems

As noted earlier, the growth rate of the internodes of deepwater rice dramatically increases in response to submergence, and part of this response is due to increased cell divisions in the intercalary meristem. To study the effect of gibberellin on the cell cycle, researchers isolated nuclei from the intercalary meristem and quantified the amount of DNA per nucleus.

In submergence-induced plants, gibberellin activates the cell division cycle first at the transition from G1 to S phase, leading to an increase in mitotic activity. To do this, gibberellin induces the expression of the genes for several **cyclin-dependent protein kinases** (**CDKs**), which are involved in regulation of the cell cycle. The transcription of these genes—first those regulating the transition from G1 to S phase, followed by those regulating the transition from G2 to M phase—is induced in the intercalary meristem by gibberellin. The result is a gibberellin induced increase in the progression from the G1 to the S phase through to mitosis and cell division.

Gibberellin Response Mutants Have Defects in Signal Transduction

Single-gene mutants impaired in their response to gibberellin provide valuable tools for identifying genes that encode possible gibberellin receptors or components of signal transduction pathways. In screenings for such

mutants, three main classes of mutations affecting plant height have been selected:
1. Gibberellin-insensitive dwarfs
2. Gibberellin-deficient mutants in which the gibberellin deficiency has been overcome by a second "suppressor" mutation, so the plants look closer to normal
3. Mutants with a constitutive gibberellin response ("slender" mutants)

All three types of gibberellin response mutants have been generated in *Arabidopsis*, but equivalent mutations have also been found in several other species; in fact, some have been in agricultural use for many years. The three types of mutant screens have sometimes identified genes encoding the same signal transduction components, even though the phenotypes being selected are completely different. This is possible because mutations at different sites in the same protein can produce vastly different phenotypes, depending on whether the mutation is in a regulatory domain or in an activity, or functional, domain. Some examples of the different phenotypes that can result from changes at different sites in the same protein are described in the sections that follow.

Functional domain (repression). The principal gibberellin signal transduction components that have been identified so far are *repressors of gibberellin signaling*; that is, they repress what we regard as gibberellin-induced tall growth and make the plant dwarf. The repressor proteins are negated or turned off by gibberellin so that the default type growth—namely, tall—is allowed to proceed. The loss of function resulting from a mutation in the functional domain of such a *negative regulator* results in the mutant appearing as if it has been treated with gibberellin; that is, it has a tall phenotype. Thus a loss-of-function mutation of
a negative regulator is like a double negative in English grammar: It translates into a positive. Because the effects of these loss-of-function mutations are pleiotropic—that is, they also affect developmental processes other than stem elongation—the steps in the pathway involved in the growth response are probably common to all gibberellin responses.

Regulatory domain. If a mutation in the gene for the same negative regulator causes a change in the *regulatory domain* (i.e., that part of the protein that receives a signal from the gibberellin receptor indicating the presence of gibberellin), the protein is unable to receive the signal, and it retains its growth-repressing activity. The phenotype of such a mutant will be that of a gibberellin-insensitive dwarf. Thus, different mutations in the same gene can give opposite phenotypes (tall versus dwarf), depending on whether the mutation is located in the repression domain or the regulatory domain.

The regulatory domain mutations that confer loss of gibberellin sensitivity result in the synthesis of a constitutively active form of the repressor than cannot be turned off by gibberellin. The more of this type of mutant repressor that is present in the cell, the more dwarf the plant will be. Hence, such regulatory domain mutations are semidominant. In contrast, mutations in the repression domain inactivate the negative regulator (i.e., they act as "knockout" alleles) so that it no longer represses growth; such mutations are recessive because in a heterozygote half the proteins will still be able to repress growth in the absence of gibberellin. *All* of the negative regulators have to be nonfunctional for the plant to grow tall without gibberellin. With this as background, we now examine specific examples of mutations in the genes that encode proteins in the gibberellin signal transduction pathway.

Different Genetic Screens Have Identified the Related Repressors GAI and RGA

Several gibberellin-insensitive dwarf mutants have been isolated from various species. The first to be isolated in *Arabidopsis* was the *gai-1* mutant. The *gai-1* mutants resemble gibberellin-deficient mutants, except that they do not respond to exogenous gibberellin. Another mutant was obtained by screening for a second mutation in a gibberellin-deficient *Arabidopsis* mutant that restores, or partially restores, wild-type growth. The original gibberellin-deficient mutant was *ga1-3,* and the second mutation that partially "rescued" the phenotype (i.e., restored normal growth) was called *rga* (for *r*epressor of *ga1-3*).4 The *rga* mutation is a recessive mutation that, when present in double copy, gives a plant of intermediate height.

Despite the contrasting phenotypes of the mutants, the wild-type *GAI* and *RGA* genes turned out to be closely related, with a very high (82%) sequence identity. The *gai-1* mutation is semidominant, as are similar gibberellin-insensitive dwarf mutations in other species.

Genetic analyses have indicated that both the GAI and RGA proteins normally act as repressors of gibberellin responses. Gibberellin acts indirectly through an unidentified signaling intermediate, which is thought to bind to the regulatory domains of the GAI and RGA proteins.

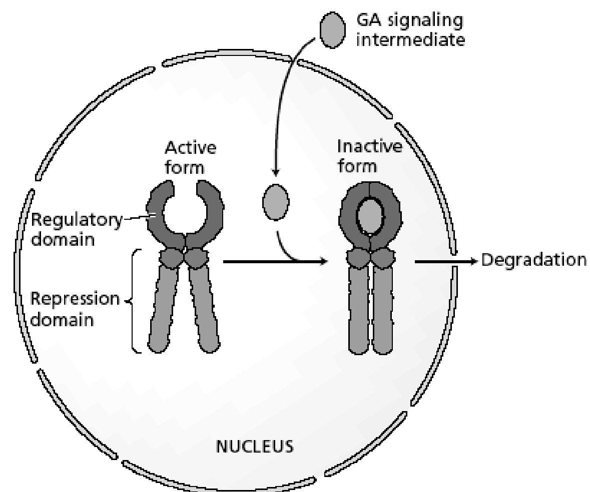


FIGURE Two main functional domains of GAI and RGA: the regulatory domain and the repression domain. The repression domain is active in the absence of gibberellin. A gibberellin-induced signaling intermediate binds to the regulatory domain, targeting it for destruction. Note that the protein forms homodimers.

The repressor is no longer able to inhibit growth, and the resulting plant is tall. The reason that *gai-1* is dwarf, while *rga* is tall, is that the mutations are in different parts of the protein. Whereas the *gai-1* mutation (which negates sensitivity of the repressor to gibberellin) is in the regulatory domain, the *rga* mutation (which prevents the action of the repressor in blocking growth) is located in the repression domain, as illustrated in figure.

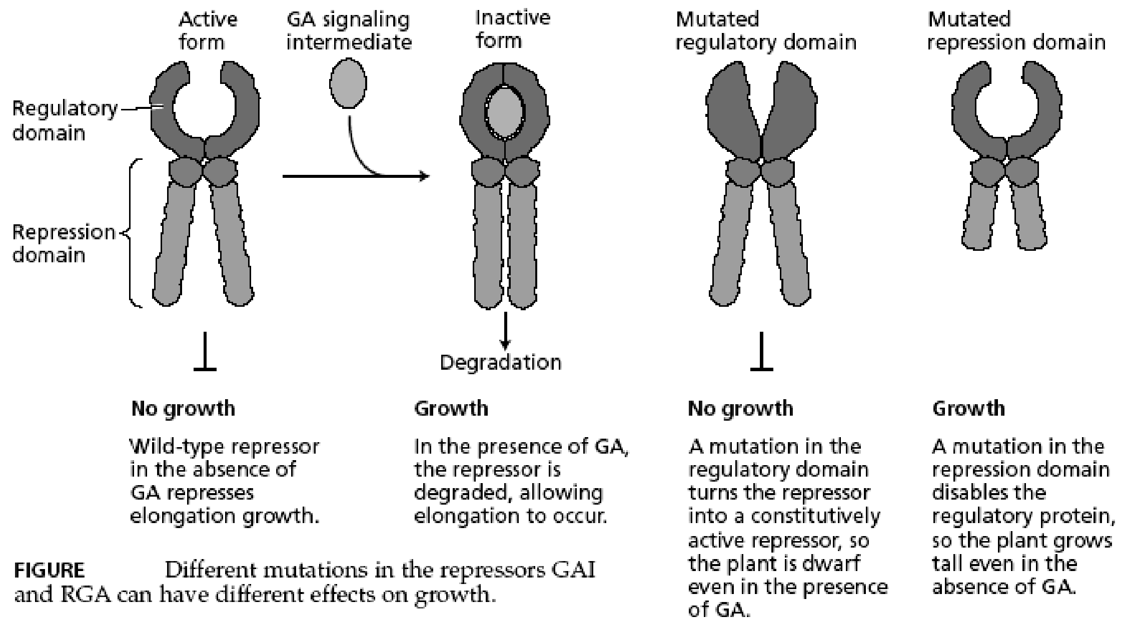


FIGURE Different mutations in the repressors GAI and RGA can have different effects on growth.

The mutant *gai-1* gene has been shown to encode a mutant protein with a deletion of 17 amino acids, which corresponds to the regulatory domain of the repressor. A similar mutation in the receptor domain of the *RGA* gene also produces a gibberellin-insensitive dwarf, demonstrating that the two related proteins have overlapping functions. Because of this deletion in the *gai-1* mutant, the action of the repressor cannot be alleviated by gibberellin, and growth is constitutively inhibited.

Gibberellins Cause the Degradation of RGA Transcriptional Repressors

The *Arabidopsis* wild-type *GAI* and *RGA* genes are members of a large gene family encoding transcriptional repressors that have highly conserved regions with nuclear localization signals. To demonstrate the nuclear localization and repressor nature of the RGA product, the *RGA* promoter was fused to the gene for a green fluorescent protein whose product can be visualized under the microscope. The green color could be seen in cell nuclei. When the plants were treated with gibberellin, there was no green color, showing that the RGA protein was not present following gibberellin treatment. However, when the gibberellin content was severely lowered by treatment with the gibberellin biosynthesis inhibitor paclobutrazol, the nuclei acquired a very intense green fluorescence, demonstrating both the presence and nuclear localization of the RGA protein only when gibberellin was absent or low.

Both GAI and RGA also have a conserved region at the amino terminus of the protein referred to as DELLA, after the code letters for the amino acids in that sequence. This region is involved in the gibberellin response because it is the location of the mutation in *gai-1* that renders it non-responsive to gibberellin. It turns out that the RGA protein is synthesized all the time; in the presence of gibberellin this protein is targeted for destruction, and the DELLA region is required for this response.
It is likely that gibberellin also brings about the turnover of GAI. *RGA* and *GAI* have partially redundant functions in maintaining the repressed state of the gibberellin signaling pathway. However, *RGA* appears to play a more dominant role than *GAI* because in a gibberellin-deficient mutant, a second mutation in the repression domain of *gai* (*gai-t6*) does not restore growth, whereas a comparable mutation in *rga* does. On the other hand, the existence of repression domain mutations in both of these genes allows for complete expression of many characteristics induced by GA, including plant height, in the absence of gibberellin.

DELLA Repressors Have Been Identified in Crop Plants

Functional DELLA repressors have been found in several crop plants that have dwarfing mutations, analogous to *gai-1*, in the genes encoding these proteins. Most notable are the *rht* (*r*educed *h*eigh*t*) mutations of wheat that have been in use in agriculture for 30 years. These alleles encode gibberellin response modulators that lack gibberellin responsiveness, leading to dwarfness.

Cereal dwarfs such as these are very important as the foundations of the green revolution that enabled large increases in yield to be obtained. Normal cereals grow too tall when close together in a field, especially with high levels of fertilizer. The result is that plants fall down (lodge), and the yield decreases concomitantly. The use of these stiff-strawed dwarf varieties that resist lodging enables high yields.

The Negative Regulator SPINDLY Is an Enzyme That Alters Protein Activity

“Slender mutants” resemble wild-type plants that have been treated with gibberellin repeatedly. They exhibit elongated internodes, parthenocarpic (seed-free) fruit growth (in dicots), and poor pollen production. Slender mutants are rare compared to dwarf mutants. One possible explanation of the slender phenotype could be simply that the mutants have higher-than-normal levels of endogenous gibberellins. For example, in the *sln* mutation of peas, a gibberellin deactivation step is blocked in the seed. As a result, the mature seed, which in the wild type contains little or no GA, has abnormally high levels of GA20. The GA20 from the seed

is then taken up by the germinating seedling and converted to the bioactive GA1, giving rise to the slender phenotype. However, once the seedling runs out of GA20 from the seed, its phenotype returns to normal.

If, on the other hand, the slender phenotype is *not* due to an overproduction of endogenous gibberellin, the mutant is considered to be a **constitutive response mutant**. The best characterized of such mutants are the ultratall mutants: *la crys* in pea, (representing mutations at two loci: *La* and *Crys*); *procera* (*pro*) in tomato; *slender* (*sln*) in barley; and *spindly* (*spy*) in *Arabidopsis*. All of these mutations are recessive and appear to be loss-of-function mutations in negative regulators of the gibberellin response pathway, as in the case of the DELLA regulators.

SPINDLY (*SPY*) in *Arabidopsis* and related genes in other species are similar in sequence to genes that encode glucosamine transferases in animals. These enzymes modify target proteins by the glycosylation of serine or threonine residues. Glycosylation can modify protein activity either directly or indirectly by interfering with or blocking sites of phosphorylation by protein kinases. The target protein for spindly proteins has not yet been identified.

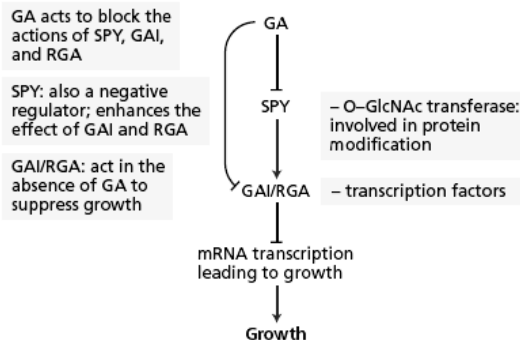


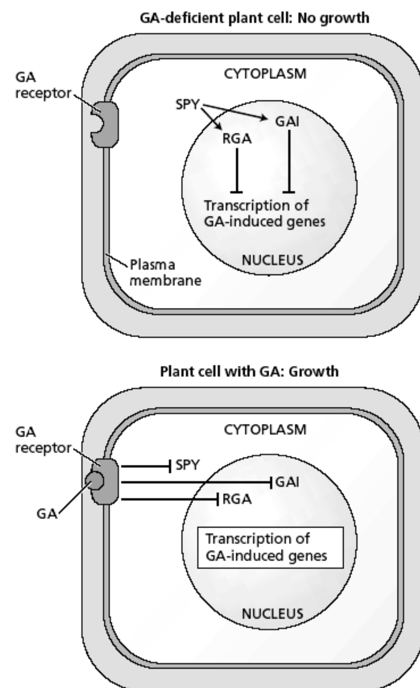
FIGURE Interactions between gibberellin and the genes *SPY, GAI,* and *RGA* in the regulation of stem elongation.

SPY Acts Upstream of *GAI* and *RGA* in the Gibberellin Signal Transduction Chain

On the basis of the evidence presented in the preceding sections and other studies on the expression of *SPY*, *GAI*, and *RGA* (Sun 2000; Dill et al. 2001), we can begin to sketch out the following elements of the gibberellin signal transduction chain (Figures):

- Two or more transcriptional regulators encoded by *GAI* and *RGA* act as inhibitors of the transcription of genes that directly or indirectly promote growth.
- SPY appears to be a signal transduction intermediate acting upstream of GAI and RGA that, itself, turns on or enhances the transcription or action of *GAI* and *RGA*, or another negative regulator.
- In the presence of gibberellin, *SPY*, *GAI*, and *RGA* are all negated or turned off.
- The RGA protein is degraded, and it is likely that GAI is similarly destroyed.

Whether gibberellin negates *GAI* and *RGA* through *SPY*, or independently, or both, is currently under investigation. However, the basic message in this case and in the cases of other plant hormones, such as ethylene and the photoreceptor phytochrome, is that the default developmental program is for the induced type of growth to occur, but the default pathway is prevented by the presence of various negative regulators.



In a GA-deficient cell in a GA biosynthesis mutant, or a wild-type cell without the GA signal, the transmembrane GA receptor is inactive in the absence of GA signal. In this situation, SPY is an active O-GlcNAc transferase that catalyzes the addition of a signal GlcNAc residue (from UDP-GlcNAc) via an O linkage to specific serine and/or threonine residues of target proteins, possibly RGA and GAI. Active RGA and GAI function as repressors of transcription, and they indirectly or directly inhibit the expression of GA-induced genes.

In the presence of GA the GA receptor is activated by binding of bioactive GA. The GA signal inhibits RGA and GAI repressors both directly and by deactivating SPY. In the absence of repression by RGA and GAI, GA-induced genes are transcribed.

FIGURE : Proposed roles of the active SPY, GAI, and RGA proteins in the GA signaling pathway within a plant cell.

Rather than directly promoting an effect, the arrival of the developmental signal—in this case gibberellin—negates the growth repressor, enabling the default condition.

GIBBERELLIN SIGNAL TRANSDUCTION: CEREAL ALEURONE LAYERS

Genetic analyses of gibberellin-regulated growth, such as the studies described in the previous section, have identified some of the genes and their gene products, but not the biochemical pathways involved in gibberellin signal transduction. The biochemical and molecular mechanisms, which are probably common to all gibberellin responses, have been studied most extensively in relation to the gibberellin- stimulated synthesis and secretion of α-amylase in cereal aleurone layers.

In this section we will describe how such studies have shed light on the location of the gibberellin receptor, the transcriptional regulation of the genes for α-amylase and other proteins, and the possible signal transduction pathways involved in the control of α-amylase synthesis and secretion by gibberellin.

Gibberellin from the Embryo Induces α-Amylase Production by Aleurone Layers

Cereal grains (*caryopses*; singular *caryopsis*) can be divided into three parts: the diploid embryo, the triploid endosperm, and the fused testa–pericarp (seed coat–fruit wall). The embryo part consists of the plant embryo proper, along with its specialized absorptive organ, the *scutellum* (plural *scutella*), which functions in absorbing the solubilized food reserves from the endosperm and transmitting them to the growing embryo. The endosperm is composed of two tissues: the centrally located starchy endosperm and the aleurone layer. The starchy endosperm, typically nonliving at maturity, consists of thin-walled cells filled with starch

grains. The aleurone layer surrounds the starchy endosperm and is cytologically and biochemically distinct from it. Aleurone cells are enclosed in thick primary cell walls and contain large numbers of protein-storing vacuoles called *protein bodies*, enclosed by a single membrane. The protein bodies also contain phytin, a mixed cation salt (mainly Mg^{2+} and K^{+}) of *myo*-inositol hexaphosphoric acid (phytic acid).

During germination and early seedling growth, the stored food reserves of the endosperm—chiefly starch and protein—are broken down by a variety of hydrolytic enzymes, and the solubilized sugars, amino acids, and other products are transported to the growing embryo. The two enzymes responsible for starch degradation are α- and β-amylase. α-Amylase hydrolyzes starch chains internally to produce oligosaccharides consisting of α-1,4-linked glucose residues. β-Amylase degrades these oligosaccharides from the ends to produce maltose, a disaccharide. Maltase then converts maltose to glucose.

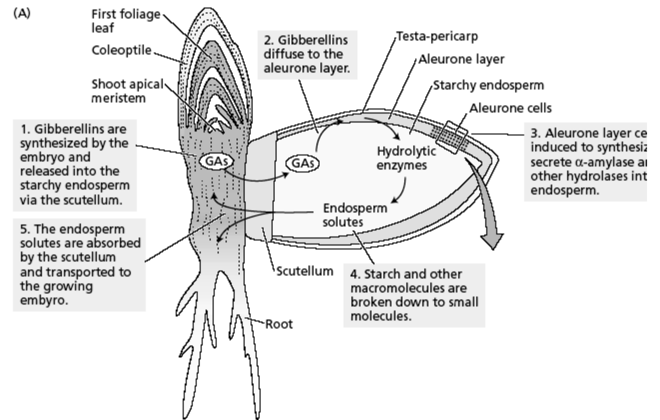
α-Amylase is secreted into the starchy endosperm of cereal seeds by both the scutellum and the aleurone layer. The sole function of the aleurone layer of the seeds of graminaceous monocots (e.g., barley, wheat, rice, rye, and oats) appears to be the synthesis and release of hydrolytic enzymes. After completing this function, aleurone cells undergo programmed cell death. Experiments carried out in the 1960s confirmed Gottlieb Haberlandt's original observation of 1890 that the secretion of starch-degrading enzymes by barley aleurone layers depends on the presence of the embryo. When the embryo was removed (i.e., the seed was de-embryonated), no starch was degraded. However, when the de-embryonated "half-seed" was incubated in close proximity to the excised embryo, starch was digested, demonstrating that the embryo produced a diffusible substance that triggered α- amylase release by the aleurone layer.

It was soon discovered that gibberellic acid (GA3) could substitute for the embryo in stimulating starch degradation. When de-embryonated half-seeds were incubated in buffered solutions containing gibberellic acid, secretion of α-amylase into the medium was greatly stimulated after an 8-hour lag period (relative to the control half-seeds incubated in the absence of gibberellic acid).

The significance of the gibberellin effect became clear when it was shown that the embryo synthesizes and releases gibberellins (chiefly GA1) into the endosperm during germination. Thus the cereal embryo efficiently regulates the mobilization of its own food reserves through the secretion of gibberellins, which stimulate the digestive function of the aleurone layer (see Figure A).

Gibberellin has been found to promote the production and/or secretion of a variety of hydrolytic enzymes that are involved in the solubilization of endosperm reserves; principal among these is α-amylase. Since the 1960s, investigators have utilized isolated aleurone layers, or even aleurone cell protoplasts, rather than half seeds. The isolated aleurone layer, consisting of a homogeneous population of target cells, provides a unique opportunity to study the molecular aspects of gibberellin action in the absence of nonresponding cell types.

In the following discussion of gibberellin-induced α- amylase production we focus on three questions:
1. How does gibberellin regulate the increase in a-amylase?
2. Where is the gibberellin receptor located in the cell?
3. What signal transduction pathways operate between the gibberellin receptor and a-amylase production?



Gibberellic Acid Enhances the Transcription of α-Amylase mRNA

Before molecular biological approaches were developed, there was already physiological and biochemical evidence that gibberellic acid might enhance α-amylase production at the level of gene transcription. The two main lines of evidence were as follows:
1. GA3-stimulated α-amylase production was shown to be blocked by inhibitors of transcription and translation.
2. Heavy-isotope- and radioactive-isotope-labeling studies demonstrated that the stimulation of α-amylase activity by gibberellin involved de novo synthesis of the enzyme from amino acids, rather than activation of preexisting enzyme.

Definitive molecular evidence now shows that gibberellin acts primarily by inducing the expression of the gene for α-amylase. It has been shown that GA3 enhances the level of translatable mRNA for α-amylase in aleurone layers. Furthermore, by using isolated nuclei, investigators also demonstrated that there was an enhanced transcription of the α-amylase gene rather than a decrease in mRNA turnover.

The purification of α-amylase mRNA, which is produced in relatively large amounts in aleurone cells, enabled the isolation of genomic clones containing both the structural gene for α-amylase and its upstream promoter sequences. These promoter sequences were then fused to the reporter gene that encodes the enzyme α-glucuronidase (GUS), which yields a blue color in the presence of an artificial substrate when the gene is expressed. The regulation of transcription by gibberellin was proved when such chimeric genes containing α-amylase promoters that were fused to reporter genes were introduced into aleurone protoplasts and the production of the blue color was shown to be stimulated by gibberellin.

The partial deletion of known sequences of bases from α-amylase promoters from several cereals indicates that the sequences conferring gibberellin responsiveness, termed *gibberellin response elements*, are located 200 to 300 base pairs upstream of the transcription start site.

A GA-MYB Transcription Factor Regulates α- Amylase Gene Expression

The stimulation of α-amylase gene expression by gibberellin is mediated by a specific transcription factor that binds to the promoter of the α-amylase gene. To demonstrate such DNA-binding proteins in rice, a technique called a *mobility shift assay* was used. This assay detects the increase in size that occurs when the α-amylase promoter binds to a protein isolated from gibberellin-treated aleurone cells.

The mobility shift assay also allowed identification of the regulatory DNA sequences (**gibberellin response elements**) in the promoter that are involved in binding the protein. Identical gibberellin response elements were found to occur in all cereal α-amylase promoters, and their presence was shown to be essential for the induction of α-amylase gene transcription by gibberellin. These studies demonstrated that gibberellin increases either the level or the activity of a transcription factor protein that switches on the production of α-amylase mRNA by binding to an upstream regulatory element in the α-amylase gene promoter. The sequence of the gibberellin response element in the α-amylase gene promoter turned out to be similar to that of the binding sites for MYB transcription factors that are known to regulate growth and development in phytochrome responses. This knowledge enabled the isolation of mRNA for a MYB transcription factor, named GA-MYB, associated with the gibberellin induction of α-amylase gene expression.

The synthesis of *GA-MYB* mRNA in aleurone cells increases within 3 hours of gibberellin application, several hours before the increase in α-amylase mRNA. The inhibitor of translation, cycloheximide, has no effect on the production of *MYB* mRNA, indicating that *GA-MYB* is a *primary response gene*, or *early gene*. In contrast, the α-amylase gene is a *secondary response gene*, or *late gene*, as indicated by the fact that its transcription is blocked by cycloheximide.

How does gibberellin cause the *MYB* gene to be expressed? Because protein synthesis is not involved, gibberellin may bring about the activation of one or more *preexisting* transcription factors. The activation of transcription factors is typically mediated by protein phosphorylation events occurring at the end of a signal transduction pathway. We will now examine what is known about the signaling pathways involved in gibberellin-induced α-amylase production up to the point of GA-MYB production.

Gibberellin Receptors May Interact with G Proteins on the Plasma Membrane

A cell surface localization of the gibberellin receptor is suggested from the fact that gibberellin that has been bound to microbeads that are unable to cross the plasma membrane is still active in inducing α-amylase production in aleurone protoplasts. In addition, microinjection of GA3 into aleurone protoplasts had no effect, but when the protoplasts were immersed in GA3 solution, they produced α-amylase. These results suggest that gibberellin acts on the outer face of the plasma membrane. Two gibberellin-binding plasma membrane proteins have been isolated through the use of purified plasma membrane and a radioactively labeled gibberellin that was chemically modified to permanently attach to protein to which it was weakly bound. Because the presence of excess gibberellin reduces binding, and these proteins from a semidwarf, gibberellin-insensitive sweet pea bind gibberellin less strongly, they may represent the gibberellin receptors.

In animal cells, heterotrimeric GTP-binding proteins (G proteins) in the cell membrane are often involved as first steps in a pathway between a hormone receptor and subsequent cytosolic signals. Evidence has been obtained that G-proteins are also involved in the early gibberellin signaling events in aleurone cells.

Treatment of oat aleurone protoplasts with a peptide called Mas7, which stimulates GTP/GDP exchange by G proteins, was found to induce α-amylase gene expression and to stimulate α-amylase secretion, suggesting that such a GTP/GDP exchange on the cell membrane is a reaction en route to the induction of α-amylase biosynthesis by gibberellin. In addition, gibberellin-induced α-amylase gene expression and secretion were inhibited by a guanine nucleotide analog that binds to the α subunit of heterotrimeric G-proteins and inhibits GTP/GDP exchange, further supporting the preceding conclusion.

Recent genetic studies have provided further support for the role of G-proteins as intermediates in the gibberellin signal transduction pathway. The rice dwarf mutant *dwarf 1* (*d1*) has a defective gene encoding the α subunit. Besides being dwarf, the aleurone layers of the *d1* mutant synthesize less α-amylase in response to gibberellin than wildtype aleurone layers do. This reduction in α-amylase production by the *d1* mutant demonstrates that G-proteins are one of the components of the gibberellin signal transduction pathway involved in both the growth response and the production of α-amylase. However, the difference in α-
amylase production between the mutant and the wild type goes away with increasing gibberellin concentration, suggesting that gibberellin can also stimulate α-amylase production by a G-protein-independent pathway.

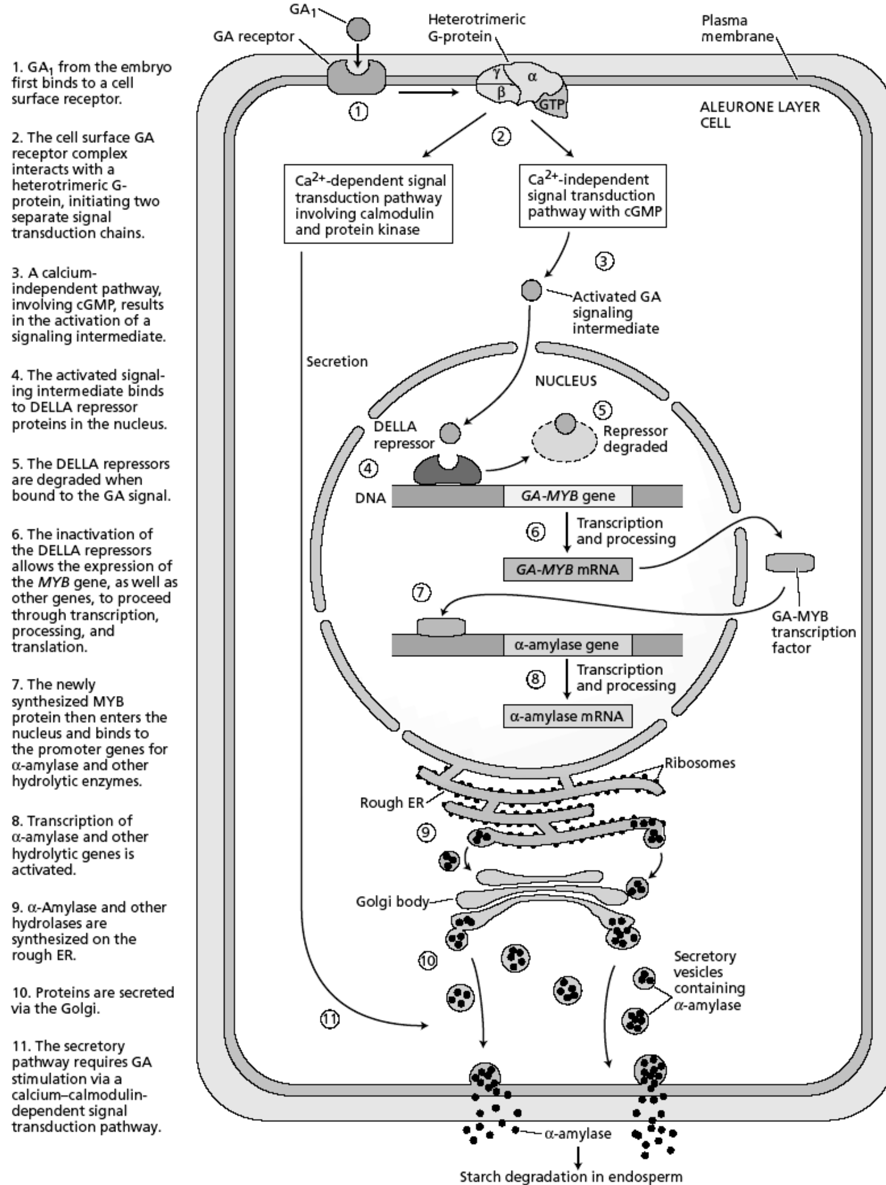
Cyclic GMP, Ca2+, and Protein Kinases Are Possible Signaling Intermediates

In animal cells, G-proteins can activate the enzyme guanylyl cyclase, the enzyme that synthesizes cGMP from GTP, leading to an increase in cGMP concetration. Cyclic GMP, in turn, can regulate ion channels, Ca2+ levels, protein kinase activity, and gene transcription. Gibberellin has been reported to cause a transient rise in cGMP levels in barley aleurone layers, suggesting a possible role for cGMP in α-amylase production.

Calcium and the calcium-binding protein calmodulin act as second messengers for many hormonal responses in animal cells, and they have been implicated in various plant responses to environmental and hormonal stimuli. The earliest event in aleurone protoplasts after the application of gibberellin is a rise in the cytoplasmic calcium concentration that occurs well before the onset of α-amylase synthesis. Without calcium, α-amylase secretion does not occur, though in barley aleurone protoplasts its synthesis goes ahead normally, so we have to conclude that, in barley, calcium is not on the signaling pathway to α-amylase gene transcription, though it does play a role in enzyme secretion.

Protein phosphorylation by protein kinases is another component in many signaling pathways, and gibberellin appears to be no exception. The injection of a protein kinase substrate into barley aleurone protoplasts to compete with endogenous protein phosphorylation inhibited α-amylase secretion, suggesting the involvement of protein phosphorylation in the α-amylase secretion pathway. This did not affect the gibberellin- stimulated increase in calcium, indicating that the protein kinase step is downstream of the calcium signaling event.

In conclusion, gibberellin signal transduction in aleurone cells seems to involve G-proteins as well as cyclic GMP, leading to production of the transcription factor GAMYB, which induces α-amylase gene transcription α-Amylase secretion has similar initial components but also involves an increase in cytoplasmic calcium and protein phosphorylation. The detailed signaling pathways remain to be worked out. A model of the known biochemical components of the gibberellin signal transduction pathways in aleurone cells is illustrated in Figure.



The Gibberellin Signal Transduction Pathway Is Similar for Stem Growth and α-Amylase Production

It is widely believed that gibberellin initially acts through a common pathway or pathways in all of its effects on development. As we have seen, the genetic approaches applied to the study of gibberellin-stimulated growth led to the identification of the *SPY/GAI/RGA* negative regulatory pathway. The proteins SPY, GAI, and RGA act as repressors of gibberellin responses. Gibberellin deactivates these repressors. Because the aleurone layers of gibberellin-insensitive dwarf wheat are also insensitive to GA, the same signal transduction pathways that regulate growth appear to regulate gibberellin-induced α-amylase production. Indeed a *SPY*-type gene associated with α-amylase production has been isolated from barley (*HvSPY*), and its expression is able to inhibit gibberellin-induced α-amylase synthesis, while GA-MYB-type factors are also implicated in the gibberellin transduction chain regulating stem growth.

Rice with the *dwarf 1* mutation also produces little α-amylase in response to gibberellin. As noted earlier, the mutation causing *dwarf 1* is known to be in the α subunit of the G-protein complex, providing evidence that the action of gibberellin in both stem elongation and the production of α-amylase are regulated by plasma membrane heterotrimeric G-proteins.

4. ETHYLENE

DURING THE NINETEENTH CENTURY, when coal gas was used for street illumination, it was observed that trees in the vicinity of streetlamps defoliated more extensively than other trees. Eventually it became apparent that coal gas and air pollutants affect plant growth and development, and ethylene was identified as the active component of coal gas.

In 1901, Dimitry Neljubov, a graduate student at the Botanical Institute of St. Petersburg in Russia, observed that dark-grown pea seedlings growing in the laboratory exhibited symptoms that were later termed the *triple response*: reduced stem elongation, increased lateral growth (swelling), and abnormal, horizontal growth. When the plants were allowed to grow in fresh air, they regained their normal morphology and rate of growth. Neljubov identified ethylene, which was present in the laboratory air from coal gas, as the molecule causing the response.

The first indication that ethylene is a natural product of plant tissues was published by H. H. Cousins in 1910. Cousins reported that "emanations" from oranges stored in a chamber caused the premature ripening of bananas when these gases were passed through a chamber containing the fruit. However, given that oranges synthesize relatively little ethylene compared to other fruits, such as apples, it is likely that the oranges used by Cousins were infected with the fungus *Penicillium*, which produces copious amounts of ethylene. In 1934, R. Gane and others identified ethylene chemically as a natural product of plant metabolism, and because of its dramatic effects on the plant it was classified as a hormone.

For 25 years ethylene was not recognized as an important plant hormone, mainly because many physiologists believed that the effects of ethylene were due to auxin, the first plant hormone to be discovered. Auxin was thought to be the main plant hormone, and ethylene was considered to play only an insignificant and indirect physiological role. Work on ethylene was also hampered by the lack of chemical techniques for its quantification. However, after gas chromatography was introduced in ethylene research in 1959, the importance of ethylene was rediscovered and its physiological significance as a plant growth regulator was recognized.

In this chapter we will describe the discovery of the ethylene biosynthetic pathway and outline some of the important effects of ethylene on plant growth and development. At the end of the chapter we will consider how ethylene acts at the cellular and molecular levels.

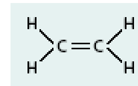
STRUCTURE, BIOSYNTHESIS, AND MEASUREMENT OF ETHYLENE

Ethylene can be produced by almost all parts of higher plants, although the rate of production depends on the type of tissue and the stage of development. In general, meristematic regions and nodal regions are the most active in ethylene biosynthesis. However, ethylene production also increases during leaf abscission and flower senescence, as well as during fruit ripening. Any type of wounding can induce ethylene biosynthesis, as can physiological stresses such as flooding, chilling, disease, and temperature or drought stress.

The amino acid methionine is the precursor of ethylene, and ACC (1-aminocyclopropane-1-carboxylic acid) serves as an intermediate in the conversion of methionine to ethylene. As we will see, the complete pathway is a cycle, taking its place among the many metabolic cycles that operate in plant cells.

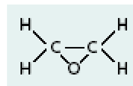
The Properties of Ethylene Are Deceptively Simple

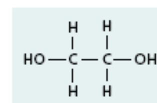
Ethylene is the simplest known olefin (its molecular weight is 28), and it is lighter than air under physiological conditions:



Ethylene

It is flammable and readily undergoes oxidation. Ethylene can be oxidized to ethylene oxide:





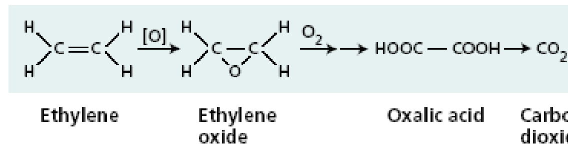
Ethylene oxide

Ethylene glycol

and ethylene oxide can be hydrolyzed to ethylene glycol:

In most plant tissues, ethylene can be completely oxidized to CO2, in the following reaction:

Complete oxidation of ethylene



Ethylene | Ethylene oxide | Oxalic acid | Carbon dioxide

Ethylene is released easily from the tissue and diffuses in the gas phase through the intercellular spaces and outside the tissue. At an ethylene concentration of 1 μL L–1 in the gas phase at 25°C, the concentration of ethylene in water is 4.4 × 10–9 *M*. Because they are easier to measure, gas phase concentrations are normally given for ethylene. Because ethylene gas is easily lost from the tissue and may affect other tissues or organs, ethylene-trapping systems are used during the storage of fruits, vegetables, and flowers. Potassium permanganate (KMnO4) is an effective absorbent of ethylene and can reduce the concentration of ethylene in apple storage areas from 250 to 10 μLL–1, markedly extending the storage life of the fruit.

Bacteria, Fungi, and Plant Organs Produce Ethylene

Even away from cities and industrial air pollutants, the environment is seldom free of ethylene because of its production by plants and microorganisms. The production of ethylene in plants is highest in senescing tissues and ripening fruits (>1.0 nL g-fresh-weight–1 h–1), but all organs of higher plants can synthesize ethylene. Ethylene is biologically active at very low concentrations—less than 1 part per million (1 μL L–1). The internal ethylene concentration in a ripe apple has been reported to be as high as 2500 μL L–1.

Young developing leaves produce more ethylene than do fully expanded leaves. In bean (*Phaseolus vulgaris*), young leaves produce 0.4 nL g–1 h–1, compared with 0.04 nL g–1 h–1 for older leaves. With few exceptions, nonsenescent tissues that are wounded or mechanically perturbed will temporarily increase their ethylene production several fold within 30 minutes. Ethylene levels later return to normal.

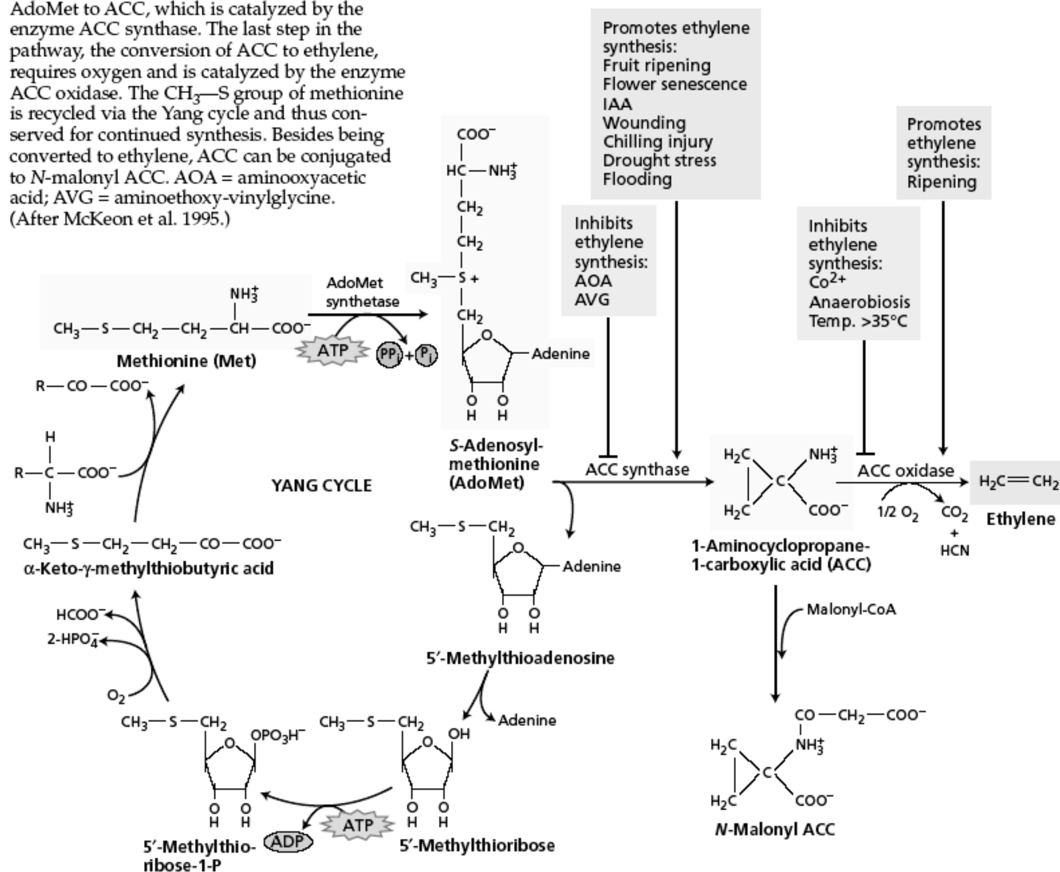


FIGURE Ethylene biosynthetic pathway and the Yang cycle. The amino acid methionine is the precursor of ethylene. The rate-limiting step in the pathway is the conversion of AdoMet to ACC, which is catalyzed by the enzyme ACC synthase. The last step in the pathway, the conversion of ACC to ethylene, requires oxygen and is catalyzed by the enzyme ACC oxidase. The CH_3—S group of methionine is recycled via the Yang cycle and thus conserved for continued synthesis. Besides being converted to ethylene, ACC can be conjugated to *N*-malonyl ACC. AOA = aminooxyacetic acid; AVG = aminoethoxy-vinylglycine. (After McKeon et al. 1995.)

Gymnosperms and lower plants, including ferns, mosses, liverworts, and certain cyanobacteria, all have shown the ability to produce ethylene. Ethylene production by fungi and bacteria contributes significantly to the ethylene content of soil. Certain strains of the common enteric bacterium *Escherichia coli* and of yeast (a fungus) produce large amounts of ethylene from methionine. There is no evidence that healthy mammalian tissues produce ethylene, nor does ethylene appear to be a metabolic product of invertebrates.

However, recently it was found that both a marine sponge and cultured mammalian cells can respond to ethylene, raising the possibility that this gaseous molecule acts as a signaling molecule in animal cells.

Regulated Biosynthesis Determines the Physiological Activity of Ethylene

In vivo experiments showed that plant tissues convert l-[14C]methionine to [14C]ethylene, and that the ethylene is derived from carbons 3 and 4 of methionine (Figure).

The CH3—S group of methionine is recycled via the Yang cycle. Without this recycling, the amount of reduced sulfur present would limit the available methionine and the synthesis of ethylene. *S*-adenosylmethionine (AdoMet), which is synthesized from methionine and ATP, is an intermediate in the ethylene biosynthetic pathway, and the immediate precursor of ethylene is **1-aminocyclopropane-1-carboxylic acid** (**ACC**) (see Figure).

The role of ACC became evident in experiments in which plants were treated with [14C]methionine. Under anaerobic conditions, ethylene was not produced from the [14C]methionine, and labeled ACC accumulated in the tissue. On exposure to oxygen, however, ethylene production surged. The labeled ACC was rapidly converted to ethylene in the presence of oxygen by various plant tissues, suggesting that ACC is the immediate precursor of ethylene in higher plants and that oxygen is required for the conversion. In general, when ACC is supplied exogenously to plant tissues, ethylene production increases substantially. This observation indicates that the synthesis of ACC is usually the biosynthetic step that limits ethylene production in plant tissues.

ACC synthase, the enzyme that catalyzes the conversion of AdoMet to ACC (see Figure), has been characterized in many types of tissues of various plants. ACC synthase is an unstable, cytosolic enzyme. Its level is regulated by environmental and internal factors, such as wounding, drought stress, flooding, and auxin. Because ACC synthase is present in such low amounts in plant tissues (0.0001% of the total protein of ripe tomato) and is very unstable, it is difficult to purify the enzyme for biochemical analysis.

ACC synthase is encoded by members of a divergent multigene family that are differentially regulated by various inducers of ethylene biosynthesis. In tomato, for example, there are at least nine ACC synthase genes, different subsets of which are induced by auxin, wounding, and/or fruit ripening.

ACC oxidase catalyzes the last step in ethylene biosynthesis: the conversion of ACC to ethylene. In tissues that show high rates of ethylene production, such as ripening fruit, ACC oxidase activity can be the rate-limiting step in ethylene biosynthesis. The gene that encodes ACC oxidase has been cloned. Like ACC synthase, ACC oxidase is encoded by a multigene family that is differentially regulated. For example, in ripening tomato fruits and senescing petunia flowers, the mRNA levels of a subset of ACC oxidase genes are highly elevated. The deduced amino acid sequences of ACC oxidases revealed that these enzymes belong to the Fe2+/ascorbate oxidase superfamily. This similarity suggested that ACC oxidase might require Fe2+ and ascorbate for activity—a requirement that has been confirmed by biochemical analysis of the protein. The low abundance of ACC oxidase and its requirement for cofactors presumably explain why the purification of this enzyme eluded researchers for so many years.

Catabolism. Researchers have studied the catabolism of ethylene by supplying $^{14}C_2H_4$ to plant tissues and tracing the radioactive compounds produced. Carbon dioxide, ethylene oxide, ethylene glycol, and the glucose conjugate of ethylene glycol have been identified as metabolic breakdown products. However, because certain cyclic olefin compounds, such as 1,4-cyclohexadiene, have been shown to block ethylene breakdown without inhibiting ethylene action, ethylene catabolism does not appear to play a significant role in regulating the level of the hormone.

Conjugation. Not all the ACC found in the tissue is converted to ethylene. ACC can also be converted to a conjugated form, *N*-malonyl ACC, which does not appear to break down and accumulates in the tissue.

A second conjugated form of ACC, 1-(γ-L-glutamylamino) cyclopropane-1-carboxylic acid (GACC), has also been identified. The conjugation of ACC may play an important role in the control of ethylene biosynthesis, in a manner analogous to the conjugation of auxin and cytokinin.

Environmental Stresses and Auxins Promote Ethylene Biosynthesis

Ethylene biosynthesis is stimulated by several factors, including developmental state, environmental conditions, other plant hormones, and physical and chemical injury. Ethylene biosynthesis also varies in a circadian manner, peaking during the day and reaching a minimum at night.

Fruit ripening. As fruits mature, the rate of ACC and ethylene biosynthesis increases. Enzyme activities for both ACC oxidase and ACC synthase increase, as do the mRNA levels for subsets of the genes encoding each enzyme. However, application of ACC to unripe fruits only slightly enhances ethylene production, indicating that an increase in the activity of ACC oxidase is the rate-limiting step in ripening.

Stress-induced ethylene production. Ethylene biosynthesis is increased by stress conditions such as drought, flooding, chilling, exposure to ozone, or mechanical wounding. In all these cases ethylene is produced by the usual biosynthetic pathway, and the increased ethylene production has been shown to result at least in part from an increase in transcription of ACC synthase mRNA. This "stress ethylene" is involved in the onset of stress responses such as abscission, senescence, wound healing, and increased disease resistance.

Auxin-induced ethylene production. In some instances, auxins and ethylene can cause similar plant responses, such as induction of flowering in pineapple and inhibition of stem elongation. These responses might be due to the ability of auxins to promote ethylene synthesis by enhancing ACC synthase activity. These observations suggest that some responses previously attributed to auxin (indole-3- acetic acid, or IAA) are in fact mediated by the ethylene produced in response to auxin.

Inhibitors of protein synthesis block both ACC and IAA-induced ethylene synthesis, indicating that the synthesis of new ACC synthase protein caused by auxins brings about the marked increase in ethylene production. Several ACC synthase genes have been identified whose transcription is elevated following application of exogenous IAA, suggesting that increased transcription is at least partly responsible for the increased ethylene production observed in response to auxin.

Posttranscriptional regulation of ethylene production.

Ethylene production can also be regulated posttranscriptionally. Cytokinins also promote ethylene biosynthesis in some plant tissues. For example, in etiolated *Arabidopsis* seedlings, application of exogenous cytokinins causes a rise in ethylene production, resulting in the tripleresponse phenotype.

Molecular genetic studies in *Arabidopsis* have shown that cytokinins elevate ethylene biosynthesis by increasing the stability and/or activity of one isoform of ACC synthase. The carboxy-terminal domain of this ACC synthase isoform appears to be the target for this posttranscriptional regulation. Consistent with this, the carboxy-terminal domain of an ACC synthase isoform from tomato has been shown to be the target for a calciumdependent phosphorylation.

Ethylene Production and Action Can Be Inhibited

Inhibitors of hormone synthesis or action are valuable for the study of the biosynthetic pathways and physiological roles of hormones. Inhibitors are particularly helpful when it is difficult to distinguish between different hormones that have identical effects in plant tissue or when a hormone affects the synthesis or the action of another hormone.

For example, ethylene mimics high concentrations of auxins by inhibiting stem growth and causing epinasty (a downward curvature of leaves). Use of specific inhibitors of ethylene biosynthesis and action made it possible to discriminate between the actions of auxin and ethylene. Studies using inhibitors showed that ethylene is the primary effector of epinasty and that auxin acts indirectly by causing a substantial increase in ethylene production.

Inhibitors of ethylene synthesis. **Aminoethoxy-vinylglycine** (**AVG**) and **aminooxyacetic acid** (**AOA**) block the conversion of AdoMet to ACC. AVG and AOA are known to inhibit enzymes that use the cofactor pyridoxal phosphate. The cobalt ion (Co^{2+}) is also an inhibitor of the ethylene biosynthetic pathway, blocking the conversion of

ACC to ethylene by ACC oxidase, the last step in ethylene biosynthesis.

Inhibitors of ethylene action. Most of the effects of ethylene can be antagonized by specific ethylene inhibitors. Silver ions (Ag^+) applied as silver nitrate ($AgNO_3$) or as silver thiosulfate ($Ag(S_2O_3)_2^{3-}$) are potent inhibitors of ethylene action. Silver is very specific; the inhibition it causes cannot be induced by any other metal ion. Carbon dioxide at high concentrations (in the range of 5 to 10%) also inhibits many effects of ethylene, such as the induction of fruit ripening, although CO_2 is less efficient than Ag^+. This effect of CO_2 has often been exploited in the storage of fruits, whose ripening is delayed at elevated CO_2 concentrations. The high concentrations of CO_2 required for inhibition make it unlikely that CO_2 acts as an ethylene antagonist under natural conditions.

The volatile compound *trans*-cyclooctene, but not its isomer *cis*-cyclooctene, is a strong competitive inhibitor of ethylene binding; *trans*-cyclooctene is thought to act by competing with ethylene for binding to the receptor. A novel inhibitor, **1-methylcyclopropene** (**MCP**), was recently found that binds almost irreversibly to the ethylene receptor. MCP shows tremendous promise in commercial applications.

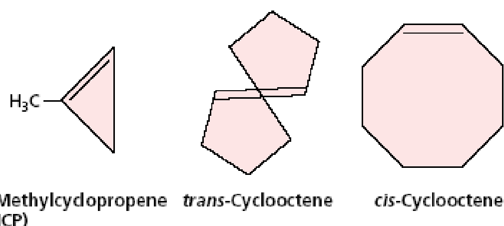


FIGURE Inhibitors that block ethylene binding to its receptor. Only the *trans* form of cyclooctene is active.

Ethylene Can Be Measured by Gas Chromatography

Historically, bioassays based on the seedling triple response were used to measure ethylene levels, but they have been replaced by **gas chromatography**. As little as 5 parts per billion (ppb) (5 pL per liter)1 of ethylene can be detected, and the analysis time is only 1 to 5 minutes.

Usually the ethylene produced by a plant tissue is allowed to accumulate in a sealed vial, and a sample is withdrawn with a syringe. The sample is injected into a gas chromatograph column in which the different gases are separated and detected by a flame ionization detector. Quantification of ethylene by this method is very accurate. Recently a novel method to measure ethylene was developed that uses a laser-driven photoacoustic detector that can detect as little as 50 parts per trillion (50 ppt = 0.05 pL L^{-1}) ethylene .

DEVELOPMENTAL AND PHYSIOLOGICAL EFFECTS OF ETHYLENE

As we have seen, ethylene was discovered in connection with its effects on seedling growth and fruit ripening. It has since been shown to regulate a wide range of responses in plants, including seed germination, cell expansion, cell differentiation, flowering, senescence, and abscission. In this section we will consider the phenotypic effects of ethylene in more detail.

Ethylene Promotes the Ripening of Some Fruits

In everyday usage, the term *fruit ripening* refers to the changes in fruit that make it ready to eat. Such changes typically include softening due to the enzymatic breakdown of the cell walls, starch hydrolysis, sugar accumulation, and the disappearance of organic acids and phenolic compounds, including tannins. From the perspective of the plant, fruit ripening means that the seeds are ready for dispersal. For seeds whose dispersal depends on animal ingestion, *ripeness* and *edibility* are synonymous. Brightly colored anthocyanins and carotenoids often accumulate in the epidermis of such fruits, enhancing their visibility. However, for seeds that rely on mechanical or other means for dispersal, *fruit ripening* may mean drying followed by splitting.

Because of their importance in agriculture, the vast majority of studies on fruit ripening have focused on edible fruits.

Ethylene has long been recognized as the hormone that accelerates the ripening of edible fruits. Exposure of such fruits to ethylene hastens the processes associated with ripening, and a dramatic increase in ethylene production accompanies the initiation of ripening. However, surveys of a wide range of fruits have shown that not all of them respond to ethylene.

All fruits that ripen in response to ethylene exhibit a characteristic respiratory rise before the ripening phase called a **climacteric**. Such fruits also show a spike of ethylene production immediately before the respiratory rise. In a smuch as treatment with ethylene induces the fruit to produce additional ethylene, its action can be described as **autocatalytic**. Apples, bananas, avocados, and tomatoes are examples of climacteric fruits. In contrast, fruits such as citrus fruits and grapes do not exhibit the respiration and ethylene production rise and are called **nonclimacteric** fruits. Other examples of climacteric and nonclimacteric fruits are given in Table 22.1.

Climacteric and nonclimacteric fruits

Climacteric	Nonclimacteric
Apple	Bell pepper
Avocado	Cherry
Banana	Citrus
Cantaloupe	Grape
Cherimoya	Pineapple
Fig	Snap bean
Mango	Strawberry
Olive	Watermelon
Peach	
Pear	
Persimmon	
Plum	
Tomato	

When unripe climacteric fruits are treated with ethylene, the onset of the climacteric rise is hastened. When nonclimacteric fruits are treated in the same way, the magnitude of the respiratory rise increases as a function of the ethylene concentration, but the treatment does not trigger production of endogenous ethylene and does not accelerate ripening.

Elucidation of the role of ethylene in the ripening of climacteric fruits has resulted in many practical applications aimed at either uniform ripening or the delay of ripening. Although the effects of exogenous ethylene on fruit ripening are straightforward and clear, establishing a causal relation

between the level of endogenous ethylene and fruit ripening is more difficult. Inhibitors of ethylene biosynthesis (such as AVG) or of ethylene action (such as CO2, MCP, or Ag+) have been shown to delay or even prevent ripening.

However, the definitive demonstration that ethylene is required for fruit ripening was provided by experiments in which ethylene biosynthesis was blocked by expression of an antisense version of either ACC synthase or ACC oxidase in transgenic tomatoes. Elimination of ethylene biosynthesis in these transgenic tomatoes completely blocked fruit ripening, and ripening was restored by application of exogenous ethylene..

Further demonstration of the requirement for ethylene in fruit ripening came from the analysis of the *never-ripe* mutation in tomato. As the name implies, this mutation completely blocks the ripening of tomato fruit. Molecular analysis revealed that *never-ripe* was due to a mutation in an ethylene receptor that rendered it unable to bind ethylene. These experiments provided unequivocal proof of the role of ethylene in fruit ripening, and they opened the door to the manipulation of fruit ripening through biotechnology.

In tomatoes several genes have been identified that are highly regulated during ripening. During tomato fruit ripening, the fruit softens as the result of cell wall hydrolysis and changes from green to red as a consequence of chlorophyll loss and the synthesis of the carotenoid pigment lycopene. At the same time, aroma and flavor components are produced.

Analysis of mRNA from tomato fruits from wild-type and transgenic tomato plants genetically engineered to lack ethylene has revealed that gene expression during ripening is regulated by at least two independent pathways:

1. *An ethylene-dependent pathway* includes genes involved in lycopene and aroma biosynthesis, respiratory metabolism, and ACC synthase.
2. *A developmental, ethylene-independent pathway* includes genes encoding ACC oxidase and chlorophyllase.

Thus, not all of the processes associated with ripening in tomato are ethylene dependent.

Leaf Epinasty Results When ACC from the Root Is Transported to the Shoot

The downward curvature of leaves that occurs when the upper (adaxial) side of the petiole grows faster than the lower (abaxial) side is termed **epinasty**. Ethylene and high concentrations of auxin induce epinasty, and it has now been established that auxin acts indirectly by inducing ethylene production.

As will be discussed later in the chapter, a variety of stress conditions, such as salt stress or pathogen infection, increase ethylene production and also induce epinasty. There is no known physiological function for the response.

In tomato and other dicots, flooding (waterlogging) or anaerobic conditions around the roots enhances the synthesis of ethylene in the shoot, leading to the epinastic response. Because these environmental stresses are sensed by the roots and the response is displayed by the shoots, a signal from the roots must be transported to the shoots. This signal is ACC, the immediate precursor of ethylene. ACC levels were found to be significantly higher in the xylem sap after flooding of tomato roots for 1 to 2 days.

Because water fills the air spaces in waterlogged soil and O2 diffuses slowly through water, the concentration of oxygen around flooded roots decreases dramatically. The elevated production of ethylene appears to be caused by the accumulation of ACC in the roots under anaerobic conditions, since the conversion of ACC to ethylene requires oxygen. The ACC accumulated in the anaerobic roots is then transported to shoots via the transpiration stream, where it is readily converted to ethylene.

Ethylene Induces Lateral Cell Expansion

At concentrations above 0.1 $\mu L\ L^{-1,}$ ethylene changes the growth pattern of seedlings by reducing the rate of elongation and increasing lateral expansion, leading to swelling of the region below the hook. These effects of ethylene are common to growing shoots of most dicots, forming part of the **triple response**. In *Arabidopsis*, the triple response consists of inhibition and swelling of the hypocotyl, inhibition of root elongation, and exaggeration of the apical hook.

The directionality of plant cell expansion is determined by the orientation of the cellulose microfibrils in the cell wall. Transverse microfibrils reinforce the cell wall in the lateral direction, so that turgor pressure is channeled into cell elongation. The orientation of the microfibrils in turn is determined by the orientation of the cortical array of microtubules in the cortical (peripheral) cytoplasm. In typical elongating plant cells, the cortical microtubules are arranged transversely, giving rise to transversely arranged cellulose microfibrils. During the seedling triple response to ethylene, the transverse pattern of microtubule alignment is disrupted, and the microtubules switch over to a longitudinal orientation. This 90° shift in microtubule orientation leads to a parallel shift in cellulose microfibril deposition. The newly deposited wall is reinforced in the longitudinal direction rather than the transverse direction, which promotes lateral expansion instead of elongation.

How do microtubules shift from one orientation to another? To study this phenomenon, pea (*Pisum sativum*) epidermal cells were injected with the microtubule protein tubulin, to which a fluorescent dye was covalently attached. The fluorescent "tag" did not interfere with the assembly of microtubules. This procedure allowed researchers to monitor the assembly of microtubules in living cells using a confocal laser scanning microscope, which can focus in many planes throughout the cell. It was found that microtubules do not reorient from the transverse to the longitudinal direction by complete depolymerization of the transverse microtubules followed by repolymerization of a new longitudinal array of microtubules.

Instead, increasing numbers of nontransversely aligned microtubules appear in particular locations. Neighboring microtubules then adopt the new alignment, so at one stage different alignments coexist before they adopt a uniformly longitudinal orientation. Although the reorientations observed in this study were spontaneous rather than induced by ethylene, it is presumed that ethylene-induced microtubule reorientation operates by a similar mechanism.

The Hooks of Dark-Grown Seedlings Are Maintained by Ethylene Production

Etiolated dicot seedlings are usually characterized by a pronounced hook located just behind the shoot apex. This hook shape facilitates penetration of the seedling through the soil, protecting the tender apical meristem.

Like epinasty, hook formation and maintenance result from ethylene-induced asymmetric growth. The closed shape of the hook is a consequence of the more rapid elongation of the outer side of the stem compared with the inner side. When the hook is exposed to white light, it opens because the elongation rate of the inner side increases, equalizing the growth rates on both sides. Red light induces hook opening, and far-red light reverses the effect of red, indicating that phytochrome is the photoreceptor involved in this process. A close interaction between phytochrome and ethylene controls hook opening. As long as ethylene is produced by the hook tissue in the dark, elongation of the cells on the inner side is inhibited. Red light inhibits ethylene formation, promoting growth on the inner side, thereby causing the hook to open.

The auxin-insensitive mutation *axr1* and treatment of wild-type seedlings with NPA (*1*-N-naphthylphthalamic acid), an inhibitor of polar auxin transport, both block the formation of the apical hook in *Arabidopsis*. These and other results indicate a role for auxin in maintaining hook structure. The more rapid growth rate of the outer tissues relative to the inner tissues could reflect an ethylene-dependent auxin gradient, analogous to the lateral auxin gradient that develops during phototropic curvature.

A gene required for formation of the apical hook, *HOOKLESS1* (so called because mutations in this gene result in seedlings lacking an apical hook), was identified in *Arabidopsis*. Disruption of this gene severely alters the pattern of expression of auxin-responsive genes. When the gene is overexpressed in *Arabidopsis*, it causes constitutive hook formation even in the light. *HOOKLESS1* encodes a putative *N*-acetyltransferase that is hypothesized to regulate—by an unknown mechanism— differential auxin distribution in the apical hook induced by ethylene.

Ethylene Breaks Seed and Bud Dormancy in Some Species

Seeds that fail to germinate under normal conditions (water, oxygen, temperature suitable for growth) are said to be dormant. Ethylene has the ability to break dormancy and initiate germination in certain seeds, such as cereals. In addition to its effect on dormancy, ethylene increases the rate of seed germination of several species. In peanuts (*Arachis hypogaea*), ethylene production and seed germination are closely correlated. Ethylene can also break bud dormancy, and ethylene treatment is sometimes used to promote bud sprouting in potato and other tubers.

Ethylene Promotes the Elongation Growth of Submerged Aquatic Species

Although usually thought of as an inhibitor of stem elongation, ethylene is able to promote stem and petiole elongation in various submerged or partially submerged aquatic plants. These include the dicots *Ranunculus sceleratus*, *Nymphoides peltata*, and *Callitriche platycarpa*, and the fern *Regnellidium diphyllum*. Another agriculturally important example is the cereal deepwater rice.

In these species, submergence induces rapid internode or petiole elongation, which allows the leaves or upper parts of the shoot to remain above water. Treatment with ethylene mimics the effects of submergence. Growth is stimulated in the submerged plants because ethylene builds up in the tissues. In the absence of O_2, ethylene synthesis is diminished, but the loss of ethylene by diffusion is retarded under water. Sufficient oxygen for growth and ethylene synthesis in the underwater parts is usually provided by aerenchyma tissue.

In deepwater rice it has been shown that ethylene stimulates internode elongation by increasing the amount of, and the sensitivity to, gibberellin in the cells of the intercalary meristem. The increased sensitivity to GA (gibberellic acid) in these cells in response to ethylene is brought about by a decrease in the level of abscisic acid (ABA), a potent antagonist of GA.

Ethylene Induces the Formation of Roots and Root Hairs

Ethylene is capable of inducing adventitious root formation in leaves, stems, flower stems, and even other roots. Ethylene has also been shown to act as a positive regulator of root hair formation in several species. This relationship has been best studied in *Arabidopsis*, in which root hairs normally are located in the epidermal cells that overlie a junction between the underlying cortical cells.

In ethylene-treated roots, extra hairs form in abnormal locations in the epidermis; that is, cells not overlying a cortical cell junction differentiate into hair cells. Seedlings grown in the presence of ethylene inhibitors (such as Ag^+), as well as ethylene-insensitive mutants, display a reduction in root hair formation in response to ethylene. These observations suggest that ethylene acts as a positive regulator in the differentiation of root hairs.

Ethylene Induces Flowering in the Pineapple Family

Although ethylene inhibits flowering in many species, it induces flowering in pineapple and its relatives, and it is used commercially in pineapple for synchronization of fruit set. Flowering of other species, such as mango, is also initiated by ethylene. On plants that have separate male and female flowers (monoecious species), ethylene may change the sex of developing flowers. The promotion of female flower formation in cucumber is one example of this effect.

Ethylene Enhances the Rate of Leaf Senescence

Senescence is a genetically programmed developmental process that affects all tissues of the plant. Several lines of physiological evidence support roles for ethylene and cytokinins in the control of leaf senescence:

• Exogenous applications of ethylene or ACC (the precursor of ethylene) accelerate leaf senescence, and
treatment with exogenous cytokinins delays leaf senescence.
• Enhanced ethylene production is associated with chlorophyll loss and color fading, which are characteristic features of leaf and flower senescence; an inverse correlation has been found between cytokinin levels in leaves and the onset of senescence.
• Inhibitors of ethylene synthesis (e.g., AVG or Co^{2+}) and action (e.g., Ag^+ or CO_2) retard leaf senescence.

Taken together, the physiological studies suggest that senescence is regulated by the balance of ethylene and cytokinin. In addition, abscisic acid (ABA) has been implicated in the control of leaf senescence.

Senescence in ethylene mutants. Direct evidence for the involvement of ethylene in the regulation of leaf senescence has come from molecular genetic studies on *Arabidopsis*. As will be discussed later in the chapter, several mutants affecting the response to ethylene have been identified. The specific bioassay employed was the tripleresponse assay in

which ethylene significantly inhibits seedling hypocotyl elongation and promotes lateral expansion.

Ethylene-insensitive mutants, such as *etr1* (*e*thylene*r*esistant *1*) and *ein2* (*e*thylene-*in*sensitive *2*), were identified by their failure to respond to ethylene (as will be described later in the chapter). The *etr1* mutant is unable to perceive the ethylene signal because of a mutation in the gene that codes for the ethylene receptor protein; the *ein2* mutant is blocked at a later step in the signal transduction pathway.

Consistent with a role for ethylene in leaf senescence, both *etr1* and *ein2* were found to be affected not only during the early stages of germination, but throughout the life cycle, including senescence. The ethylene mutants retained their chlorophyll and other chloroplast components for a longer period of time compared to the wild type. However, because the total life spans of these mutants were increased by only 30% over that of the wild type, ethylene appears to increase the *rate* of senescence, rather than acting as a developmental switch that initiates the senescence process.

Use of genetic engineering to probe senescence. Another very useful genetic approach that offers direct evidence for the function of specific gene(s) is based on transgenic plants. Through genetic engineering technology, the roles of both ethylene and cytokinins in the regulation of leaf senescence have been confirmed.

One way to suppress the expression of a gene is to transform the plant with antisense DNA, which consists of the gene of interest in the reverse orientation with respect to the promoter. When the antisense gene is transcribed, the resulting antisense mRNA is complementary to the sense mRNAand will hybridize to it. Because double-stranded RNA is rapidly degraded in the cell, the effect of the antisense gene is to deplete the cell of the sense mRNA.

Transgenic plants expressing antisense versions of genes that encode enzymes involved in the ethylene biosynthetic pathway, such as ACC synthase and ACC oxidase, can synthesize ethylene only at very low levels. Consistent with a role for ethylene in senescence, such antisense mutants have been shown to exhibit delayed leaf senescence, as well as fruit ripening, in tomato.

The Role of Ethylene in Defense Responses Is Complex

Pathogen infection and disease will occur only if the interactions between host and pathogen are genetically compatible. However, ethylene production generally increases in response to pathogen attack in both compatible (i.e., pathogenic) and noncompatible (nonpathogenic) interactions. The discovery of ethylene-insensitive mutants has allowed the role of ethylene in the response to various pathogens to be assessed. The emerging picture is that the involvement of ethylene in pathogenesis is complex and depends on the particular host–pathogen interaction. For example, blocking the ethylene response does not affect the resistance response to *Pseudomonas* bacteria in *Arabidopsis* or to tobacco mosaic virus in tobacco. In compatible interactions of these pathogens and hosts, however, elimination of ethylene responsiveness prevents the development of disease symptoms, even though the growth of the pathogen appears to be unaffected.

On the other hand, ethylene, in combination with jasmonic acid, is required for the activation of several plant defense genes. In addition, ethylene-insensitive tobacco and *Arabidopsis* mutants become susceptible to several necrotrophic (cell-killing) soil fungal pathogens that are normally not plant pathogens. Thus, ethylene appears to be involved in the resistance response to some pathogens, but not others.

Ethylene Biosynthesis in the Abscission Zone Is Regulated by Auxin

The shedding of leaves, fruits, flowers, and other plant organs is termed **abscission**. Abscission takes place in specific layers of cells, called **abscission layers**, which become morphologically and biochemically differentiated during organ development. Weakening of the cell walls at the abscission layer depends on cell wall–degrading enzymes such as cellulase and polygalacturonase.

The ability of ethylene gas to cause defoliation in birch trees. The wild-type tree on the left has lost all its leaves. The tree on the right has been transformed with a gene for the *Arabidopsis* ethylene receptor ETR1-1 carrying a dominant mutation (discussed in the next section). This tree is unable to respond to ethylene and does not shed its leaves after ethylene treatment.

Ethylene appears to be the primary regulator of the abscission process, with auxin acting as a suppressor of the ethylene effect. However, supraoptimal auxin concentrations stimulate ethylene production, which has led to the use of auxin analogs as defoliants. For example, 2,4,5-T, the active ingredient in Agent Orange, was widely used as a defoliant during the Vietnam War. Its action is based on its ability to increase ethylene biosynthesis, thereby stimulating leaf abscission.

A model of the hormonal control of leaf abscission describes the process in three distinct sequential phases:
1. Leaf maintenance phase. Prior to the perception of any signal (internal or external) that initiates the abscission process, the leaf remains healthy and fully functional in the plant. A gradient of auxin from the blade to the stem maintains the abscission zone in a nonsensitive state.
2. Shedding induction phase. A reduction or reversal in the auxin gradient from the leaf, normally associated with leaf senescence, causes the abscission zone to become sensitive to ethylene. Treatments that enhance leaf senescence may promote abscission by interfering with auxin synthesis and/or transport in the leaf.
3. Shedding phase. The sensitized cells of the abscission zone respond to low concentrations of endogenous ethylene by synthesizing and secreting cellulase and other cell wall–degrading enzymes, resulting in shedding.

During the early phase of leaf maintenance, auxin from the leaf prevents abscission by maintaining the cells of the abscission zone in an ethylene-insensitive state. It has long been known that removal of the leaf blade (the site of auxin production) promotes petiole abscission. Application of exogenous auxin to petioles from which the leaf blade has been removed delays the abscission process. However, application of auxin to the proximal side of the abscission zone (i.e., the side closest to the stem) actually *accelerates* the abscission process. These results indicate that it is not the absolute amount of auxin at the abscission zone, but rather the auxin *gradient*, that controls the ethylene sensitivity of these cells.

In the shedding induction phase, the amount of auxin from the leaf decreases and the ethylene level rises. Ethylene

appears to decrease the activity of auxin both by reducing its synthesis and transport and by increasing its destruction. The reduction in the concentration of free auxin increases the response of specific target cells to ethylene.

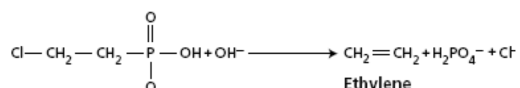
The shedding phase is characterized by the induction of genes encoding specific hydrolytic enzymes of cell wall polysaccharides and proteins. The *target cells*, located in the abscission zone, synthesize cellulase and other polysaccharide-degrading enzymes, and secrete them into the cell wall via secretory vesicles derived from the Golgi. The action of these enzymes leads to cell wall loosening, cell separation, and abscission.

Ethylene Has Important Commercial Uses

Because ethylene regulates so many physiological processes in plant development, it is one of the most widely used plant hormones in agriculture. Auxins and ACC can trigger the natural biosynthesis of ethylene and in several cases are used in agricultural practice. Because of its high diffusion rate, ethylene is very difficult to apply in the field as a gas, but this limitation can be overcome if an ethylene-releasing compound is used. The most widely used such compound is ethephon, or 2- chloroethylphosphonic acid, which was discovered in the 1960s and is known by various trade names, such as Ethrel.

Ethephon is sprayed in aqueous solution and is readily absorbed and transported within the plant. It releases ethylene slowly by a chemical reaction, allowing the hormone to exert its effects: Ethephon hastens fruit ripening of apple and tomato and degreening of citrus, synchronizes flowering and fruit set in pineapple, and accelerates abscission of flowers and fruits. It can be used to induce fruit thinning or fruit drop in cotton, cherry, and walnut. It is also used to promote female sex expression in cucumber, to prevent self- pollination and increase yield, and to inhibit terminal growth of some plants in order to promote lateral growth and compact flowering stems.

Storage facilities developed to inhibit ethylene production and promote preservation of fruits have a controlled atmosphere of low O_2 concentration and low temperature that inhibits ethylene biosynthesis. A relatively high concentration of CO2 (3 to 5%) prevents ethylene's action as a ripening promoter. Low pressure (vacuum) is used to remove ethylene and oxygen from the storage chambers, reducing the rate of ripening and preventing overripening. Specific inhibitors of ethylene biosynthesis and action are also useful in postharvest preservation. Silver (Ag+) is used extensively to increase the longevity of cut carnations and several other flowers. The potent inhibitor AVG retards fruit ripening and flower fading, but its commercial use has not yet been approved by regulatory agencies. The strong,



offensive odor of *trans*-cyclooctene precludes its use in agriculture. Currently, 1-methylcyclopropene (MCP) is being developed for use in a variety of postharvest applications. The near future may see a variety of agriculturally important species that have been genetically modified to manipulate the biosynthesis of ethylene or its perception. The inhibition of ripening in tomato by expression of an antisense version of ACC synthase and ACC oxidase has already been mentioned. Another example of this technology is in petunia, in which ethylene biosynthesis has been blocked by transformation of an antisense version of ACC oxidase. Senescence and petal wilting of cut flowers are delayed for weeks in these transgenic plants.

CELLULAR AND MOLECULAR MODES OF ETHYLENE ACTION

Despite the broad range of ethylene's effects on development, the primary steps in ethylene action are assumed to be similar in all cases: They all involve binding to a receptor, followed by activation of one or more signal transduction pathways leading to the cellular response. Ultimately, ethylene exerts its effects primarily by altering the pattern of gene expression. In recent years, remarkable progress has been made in our understanding of ethylene perception, as the result of molecular genetic studies of *Arabidopsis thaliana*.

One key to the elucidation of ethylene signaling components has been the use of the triple-response morphology of etiolated *Arabidopsis* seedlings to isolate mutants affected in their response to ethylene. Two classes of mutants have been identified by experiments in which mutagenized *Arabidopsis* seeds were grown on an agar medium in the presence or absence of ethylene for 3 days in the dark:

1. Mutants that fail to respond to exogenous ethylene (ethylene-resistant or ethylene-insensitive mutants)
2. Mutants that display the response even in the absence of ethylene (constitutive mutants)

Ethylene-insensitive mutants are identified as tall seedlings extending above the lawn of short, tripleresponding seedlings when grown in the presence of ethylene. Conversely, constitutive ethylene response mutants are identified as seedlings displaying the triple response in the absence of exogenous ethylene.

Ethylene Receptors Are Related to Bacterial Two-Component System Histidine Kinases

The first ethylene-insensitive mutant isolated was *etr1* (*e*thylene-*r*esistant *1*). The *etr1* mutant was identified in a screen for mutations that block the response of *Arabidopsis* seedlings to ethylene. The amino acid sequence of the carboxy-terminal half of *ETR1* is similar to bacterial two-component histidine kinases—receptors used by bacteria to perceive various environmental cues, such as chemo-sensory stimuli, phosphate availability, and osmolarity.

Bacterial two-component systems consist of a sensor histidine kinase and a response regulator, which often acts as a transcription factor. ETR1 was the first example of a eukaryotic histidine kinase, but others have since been found in yeast, mammals, and plants. Both phytochrome and the cytokinin receptor also share sequence similarity to bacterial two-component histidine kinases.

The similarity to bacterial receptors and the ethylene insensitivity of the *etr1* mutants suggested that ETR1 might be an ethylene receptor. Consistent with this hypothesis, *ETR1* expression in yeast conferred the ability to bind radiolabeled ethylene with an affinity that closely parallels the dose-response curve of *Arabidopsis* seedlings to ethylene.

The *Arabidopsis* genome encodes four additional proteins similar to ETR1 that also function as ethylene receptors:

ETR2, ERS1 (*E*TR1-*r*elated *s*equence *1*), ERS2, and EIN4. Like ETR1, these receptors have been shown to bind ethylene, and missense mutations in the genes that encode these proteins, analogous to the original *etr1* mutation, prevent ethylene binding to the receptor while allowing the receptor to function normally as a regulator of the ethylene response pathway in the absence of ethylene.

All of these proteins share at least two domains:
1. The amino-terminal domain spans the membrane at least three times and contains the ethylene-binding site. Ethylene can readily access this site because of its hydrophobicity.
2. The middle portion of the ethylene receptors contains a histidine kinase catalytic domain.

A subset of the ethylene receptors also have a carboxyterminal domain that is similar to bacterial two-component receiver domains. In other two-component systems, binding of ligand regulates the activity of the histidine kinase domain, which autophosphorylates a conserved histidine residue. The phosphate is then transferred to an aspartic acid residue located within the fused receiver domain. Although histidine kinase activity has been demonstrated for one of the ethylene receptors—ETR1—several others are missing critical amino acids, making it unlikely that they possess histidine kinase activity. Thus the biochemical mechanism of these ethylene receptors is not known.

Recent studies indicate that ETR1 is located on the *endoplasmic reticulum*, rather than on the plasma membrane as originally assumed. Such an intracellular location for the ethylene receptor is consistent with the hydrophobic nature of ethylene, which enables it to pass freely through the plasma membrane into the cell. In this respect ethylene is similar to the hydrophobic signaling molecules of animals, such as steroids and the gas nitric oxide, which also bind to intracellular receptors.

High-Affinity Binding of Ethylene to Its Receptor Requires a Copper Cofactor

Even prior to the identification of its receptor, scientists had predicted that ethylene would bind to its receptor via a transition metal cofactor, most likely copper or zinc. This prediction was based on the high affinity of olefins, such as ethylene, for these transition metals. Recent genetic and biochemical studies have borne out these predictions.

Analysis of the ETR1 ethylene receptor expressed in yeast demonstrated that a copper ion was coordinated to the protein and that this copper was necessary for highaffinity ethylene binding. Silver ion could substitute for copper to yield high-affinity binding, which indicates that silver blocks the action of ethylene not by interfering with ethylene binding, but by preventing the changes in the protein that normally occur when ethylene binds to the receptor.

Evidence that copper binding is required for ethylene receptor function in vivo came from identification of the *RAN1* gene in *Arabidopsis*. Strong *ran1* mutations block the formation of functional ethylene receptors. Cloning of *RAN1* revealed that it encodes a protein similar to a yeast protein required for the transfer of a copper ion cofactor to an iron transport protein. In an analogous manner, RAN1 is likely to be involved in the addition of a copper ion cofactor necessary for the function of the ethylene receptors.

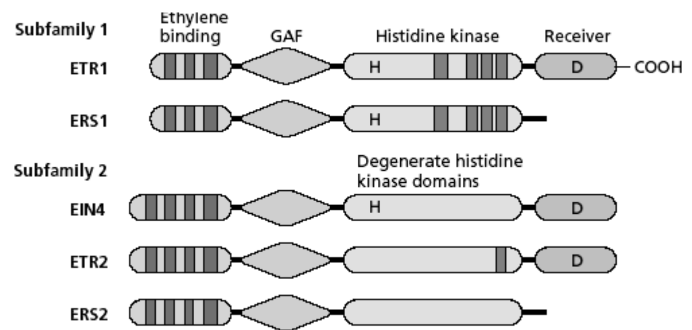


FIGURE Schematic diagram of five ethylene receptor proteins and their functional domains. The GAF domain is a conserved cGMP-binding domain found in a diverse group of proteins. Note that EIN4, ETR2, and ERS2 have degenerate histidine kinase domains.

Unbound Ethylene Receptors Are Negative Regulators of the Response Pathway

In *Arabidopsis*, tomato, and probably most other plant species, the ethylene receptors are encoded by multigene families. Targeted disruption (complete inactivation) of the five *Arabidopsis* ethylene receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) has revealed that they are functionally redundant. That is, disruption of any single gene encoding one of these proteins has no effect, but a plant with disruptions in all five receptor genes exhibits a constitutive ethylene response phenotype.

The observation that ethylene responses, such as the triple response, become constitutive when the receptors are disrupted indicates that the receptors are normally "on" (i.e., in the active state) in the *absence* of ethylene, and that the function of the receptor *minus* its ligand (ethylene), is to *shut off* the signaling pathway that leads to the response. Binding of ethylene turns off the receptors, thus allowing the response pathway to proceed.

This somewhat counterintuitive model for ethylene receptors as negative regulators of a signaling pathway is unlike the mechanism of most animal receptors, which, after binding their ligands, serve as positive regulators of their respective signal transduction pathways. In contrast to the disrupted receptors, receptors with missense mutations at the ethylene binding site (as occurs in the original *etr1* mutant) are unable to bind ethylene, but are still active as negative regulators of the ethylene response pathway. Such missense mutations result in a plant that expresses a subset of receptors that can no longer be turned off by ethylene, and thus confer a *dominant ethylene-insensitive phenotype*. Even though the normal receptors can all be turned off by ethylene, the mutant receptors continue to signal the cell to suppress ethylene responses whether ethylene is present or not.

A Serine/Threonine Protein Kinase Is Also Involved in Ethylene Signaling

The recessive *ctr1* (*c*onstitutive *t*riple *r*esponse *1* = triple response in the absence of ethylene) mutation was identified in screens for mutations that constitutively activated ethylene responses. The fact that the mutation caused an *activation* of the ethylene response suggests that the wild-type protein also acts as a *negative regulator* of the response pathway, similar to the ethylene receptors.

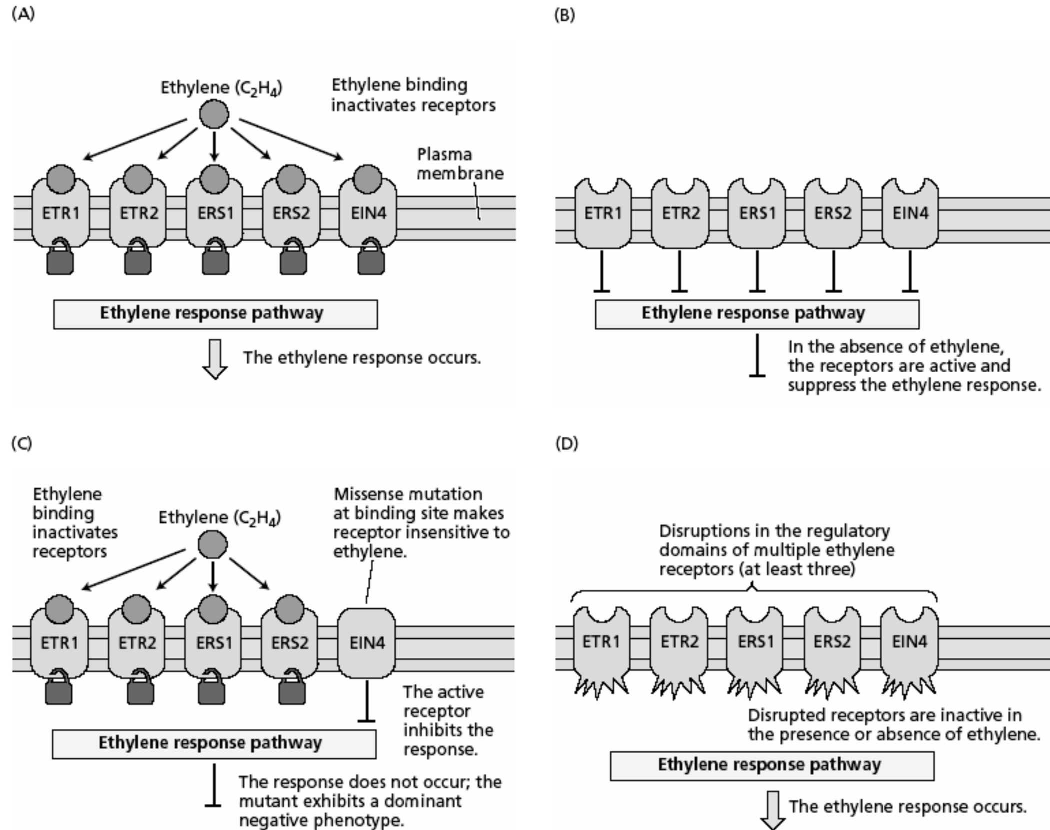


FIGURE Model for ethylene receptor action based on the phenotype of receptor mutants. (A) In the wild type, ethylene binding inactivates the receptors, allowing the response to occur. (B) In the absence of ethylene the receptors act as negative regulators of the response pathway. (C) A missense mutation that interferes with ethylene binding to its receptor, but leaves the regulatory site active, results in a dominant negative phenotype. (D) Disruption mutations in the regulatory sites result in a constitutive ethylene response.

CTR1 appears to be related to RAF-1, a MAPKKK serine/ threonine protein kinase (*m*itogen-*a*ctivated *p*rotein *k*inase *k*inase *k*inase) that is involved in the transduction of various external regulatory signals and developmental signaling pathways in organisms ranging from yeast to humans. In animal cells, the final product in the MAP kinase cascade is a phosphorylated transcription factor that regulates gene expression in the nucleus.

EIN2 Encodes a Transmembrane Protein

The *ein2* (*e*thylene-*in*sensitive *2*) mutation blocks all ethylene responses in both seedling and adult *Arabidopsis* plants. The *EIN2* gene encodes a protein containing 12 membranespanning domains that is most similar to the N-RAMP (*n*atural *r*esistance–*a*ssociated *m*acrophage *p*rotein) family of cation transporters in animals, suggesting that it may act as a channel or pore. To date, however, researchers have failed to demonstrate a transport activity for this protein, and the intracellular location of the protein is not known.

Interestingly, mutations in the *EIN2* gene have also been identified in genetic screens for resistance to other hormones, such as jasmonic acid and ABA, suggesting that EIN2 may be a common intermediate in the signal transduction pathways of various hormones and other chemical signals.

Ethylene Regulates Gene Expression

One of the primary effects of ethylene signaling is an alteration in the expression of various target genes. Ethylene affects the mRNA transcript levels of numerous genes, including the genes that encode cellulase, as well as ripening- related genes and ethylene biosynthesis genes. Regulatory sequences called **ethylene response elements**, or **EREs**, have been identified from the ethylene-regulated genes. Key components mediating ethylene's effects on gene expression are the EIN3 family of transcription factors. There are at least four *EIN3*-like genes in *Arabidopsis*, and homologs have been identified in both tomato and tobacco. In response to an ethylene signal, homodimers of EIN3 or its paralogs (closely related proteins), bind to the promoter of a gene called *ERF1* (*e*thylene *r*esponse *f*actor *1*) and activate its transcription.

ERF1 encodes a protein that belongs to the **ERE-binding protein** (**EREBP**) family of transcription factors, which were first identified in tobacco as proteins that bind to ERE sequences. Several EREBPs are rapidly up-regulated in response to ethylene. The EREBP genes exist in *Arabidopsis* as a very large gene family, but only a few of the genes are inducible by ethylene.

Genetic Epistasis Reveals the Order of the Ethylene Signaling Components

The order of action of the genes *ETR1*, *EIN2*, *EIN3*, and *CTR1* has been determined by the analysis of how the mutations interact with each other (i.e., their epistatic order). Two mutants with opposite phenotypes are crossed, and a line harboring both mutations (the double mutant) is identified in the F2 generation. In the case of the ethylene response mutants, researchers constructed a line doubly mutant for *ctr1*, a constitutive ethylene response mutant, and one of the ethylene-insensitive mutations.

The phenotype that the double mutant displays reveals which of the mutations is epistatic to the other. For example, if an *etr1/ctr1* double mutant displays a *ctr1* mutant phenotype, the *ctr1* mutation is said to be epistatic to *etr1*. From this it can be inferred that CTR1 acts downstream of ETR1. In this way, the order of action of *ETR1*, *EIN2*, and *EIN3* were determined relative to *CTR1*.

The ETR1 protein has been shown to interact physically with the predicted downstream protein, CTR1, suggesting that the ethylene receptors may directly regulate the kinase activity of CTR1. The model in Figure summarizes these and other data. Genes that are similar to several of these *Arabidopsis* signaling genes have been found in other species .

This model is still incomplete because other ethylene response mutations have been identified that act in this pathway. In addition, we are only beginning to understand the biochemical properties of these proteins and how they interact. However, we are beginning to glimpse the outline of the molecular basis for the perception and transduction of this hormonal signal.

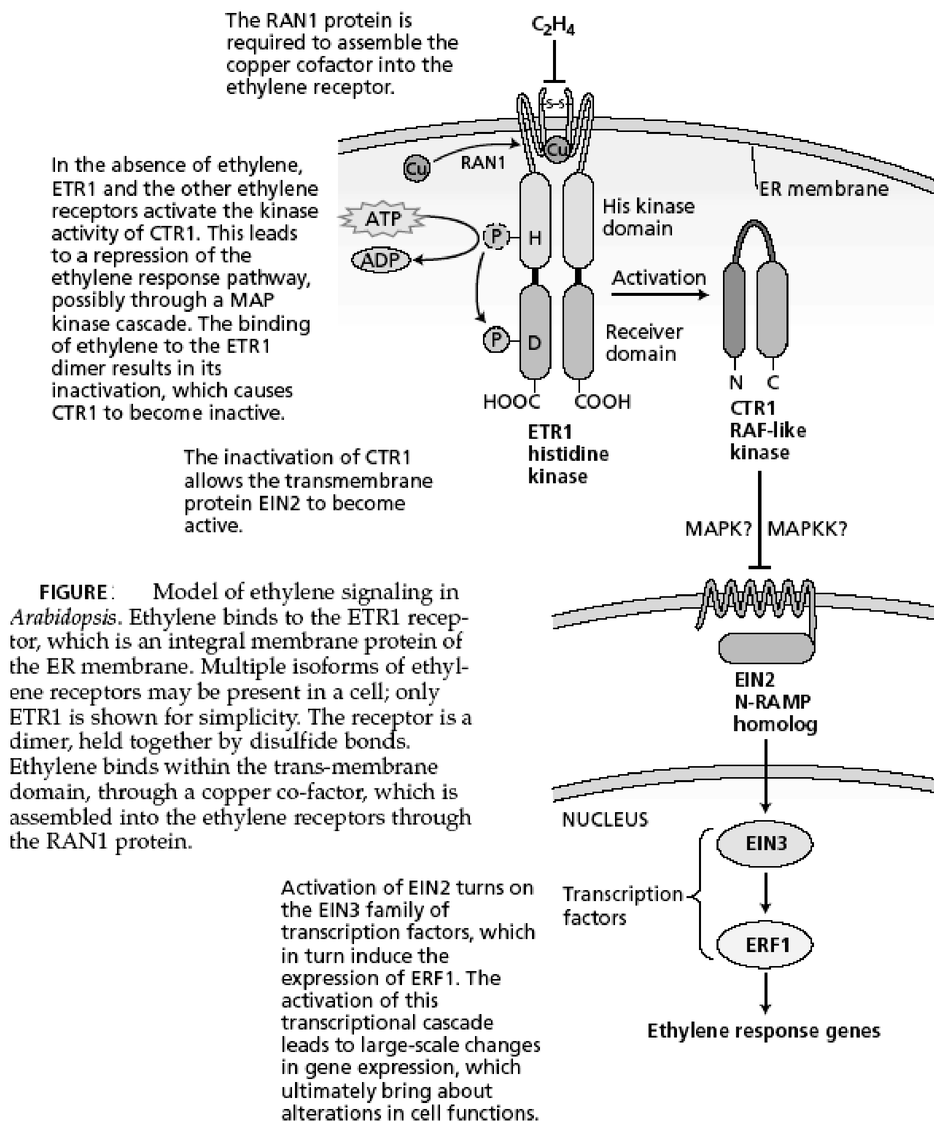


FIGURE : Model of ethylene signaling in *Arabidopsis*. Ethylene binds to the ETR1 receptor, which is an integral membrane protein of the ER membrane. Multiple isoforms of ethylene receptors may be present in a cell; only ETR1 is shown for simplicity. The receptor is a dimer, held together by disulfide bonds. Ethylene binds within the trans-membrane domain, through a copper co-factor, which is assembled into the ethylene receptors through the RAN1 protein.

5. ABSCISSIC ACID

THE EXTENT AND TIMING OF PLANT GROWTH are controlled by the coordinated actions of positive and negative regulators. Some of the most obvious examples of regulated nongrowth are seed and bud dormancy, adaptive features that delay growth until environmental conditions are favorable. For many years, plant physiologists suspected that the phenomena of seed and bud dormancy were caused by inhibitory compounds, and they attempted to extract and isolate such compounds from a variety of plant tissues, especially dormant buds.

Early experiments used paper chromatography for the separation of plant extracts, as well as bioassays based on oat coleoptile growth. These early experiments led to the identification of a group of growth-inhibiting compounds, including a substance known as *dormin* purified from sycamore leaves collected in early autumn, when the trees were entering dormancy. Upon discovery that dormin was chemically identical to a substance that promotes the abscission of cotton fruits, *abscisin II*, the compound was renamed **abscisic acid** (**ABA**), to reflect its supposed involvement in the abscission process.

It is now known that ethylene is the hormone that triggers abscission and that ABA-induced abscission of cotton fruits is due to ABA's ability to stimulate ethylene production. As will be discussed in this chapter, ABAis now recognized as an important plant hormone in its own right. It inhibits growth and stomatal opening, particularly when the plant is under environmental stress. Another important function is its regulation of seed maturation and dormancy. In retrospect, *dormin* would have been a more appropriate name for this hormone, but the name *abscisic acid* is firmly entrenched in the literature.

OCCURRENCE, CHEMICAL STRUCTURE, AND MEASUREMENT OF ABA

Abscisic acid has been found to be a ubiquitous plant hormone in vascular plants. It has been detected in mosses but appears to be absent in liverworts. Several genera of fungi make ABA as a secondary metabolite. Within the plant, ABA has been detected in every major organ or living tissue from the root cap to the apical bud. ABA is synthesized in almost all cells that contain chloroplasts or amyloplasts.

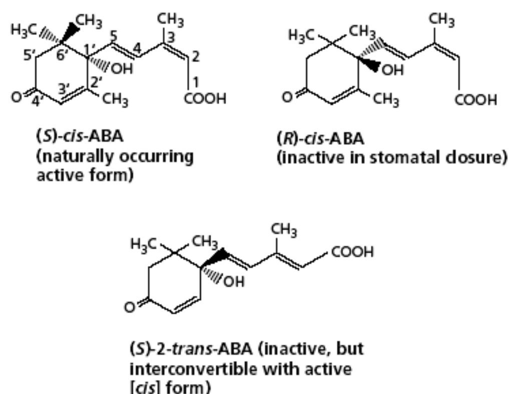


FIGURE The chemical structures of the *S* (counterclockwise array) and *R* (clockwise array) forms of *cis*-ABA, and the (*S*)-2-*trans* form of ABA. The numbers in the diagram of (*S*)-*cis*-ABA identify the carbon atoms.

The Chemical Structure of ABA Determines Its Physiological Activity

ABA is a 15-carbon compound that resembles the terminal portion of some carotenoid molecules (Figure). The orientation of the carboxyl group at carbon 2 determines the *cis* and *trans* isomers of ABA. Nearly all the naturally occurring ABA is in the *cis* form, and by convention the name *abscisic acid* refers to that isomer.

ABA also has an asymmetric carbon atom at position 1′in the ring, resulting in the *S* and *R* (or + and –, respectively) enantiomers. The *S* enantiomer is the natural form; commercially available synthetic ABA is a mixture of approximately equal amounts of the *S* and *R* forms. The *S* enantiomer is the only one that is active in fast responses to ABA, such as stomatal closure. In long-term responses, such as seed maturation, both enantiomers are active. In contrast to the *cis* and *trans* isomers, the *S* and *R* forms cannot be interconverted in the plant tissue.

Studies of the structural requirements for biological activity of ABA have shown that almost any change in the molecule results in loss of activity.

ABA Is Assayed by Biological, Physical, and Chemical Methods

Avariety of bioassays have been used for ABA, including inhibition of coleoptile growth, germination, or GAinduced α-amylase synthesis. Alternatively, promotion of stomatal closure and gene expression are examples of rapid inductive responses.

Physical methods of detection are much more reliable than bioassays because of their specificity and suitability for quantitative analysis. The most widely used techniques are those based on gas chromatography or high-performance liquid chromatography (HPLC). Gas chromatography allows detection of as little as 10–13 g ABA, but it requires several preliminary purification steps, including thin-layer chromatography. Immunoassays are also highly sensitive and specific.

BIOSYNTHESIS, METABOLISM, AND TRANSPORT OF ABA

As with the other hormones, the response to ABA depends on its concentration within the tissue and on the sensitivity of the tissue to the hormone. The processes of biosynthesis, catabolism, compartmentation, and transport all contribute to the concentration of active hormone in the tissue at any given stage of development. The complete biosynthetic pathway of ABA was elucidated with the aid of ABA-deficient mutants blocked at specific steps in the pathway.

ABA Is Synthesized from a Carotenoid Intermediate

ABA biosynthesis takes place in chloroplasts and other plastids via the pathway depicted in Figure. Several ABA-deficient mutants have been identified with lesions at specific steps of the pathway. These mutants exhibit abnormal phenotypes that can be corrected by the application of exogenous ABA. For example, *flacca* (*flc*) and *sitiens* (*sit*) are "wilty mutants" of tomato in which the tendency of the leaves to wilt (due to an inability to close their stomata) can be prevented by the application of exogenous ABA. The *aba* mutants of *Arabidopsis* also exhibit a wilty phenotype. These and other mutants have been useful in elucidating the details of the pathway.

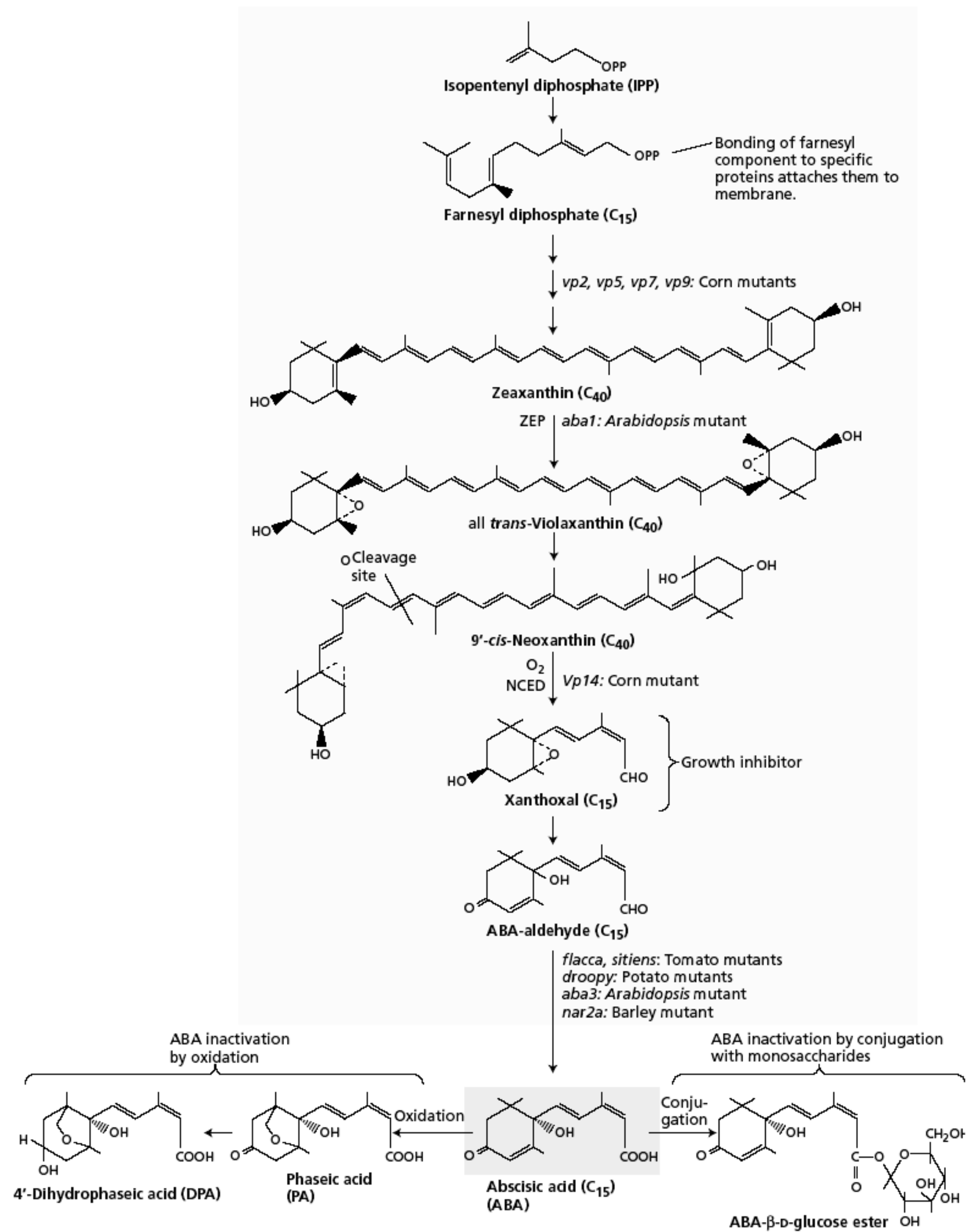


FIGURE . ABA biosynthesis and metabolism. In higher plants, ABA is synthesized via the terpenoid pathway . Some ABA-deficient mutants that have been helpful in elucidating the pathway are shown at the steps at which they are blocked. The pathways for ABA catabolism include conjugation to form ABA-β-D-glucosyl ester or oxidation to form phaseic acid and then dihydrophaseic acid. ZEP = zeaxanthin epoxidase; NCED = 9-cis-epoxy-carotenoids dioxygenase.

The pathway begins with isopentenyl diphosphate (IPP), the biological isoprene unit, and leads to the synthesis of the C40 xanthophyll (i.e., oxygenated carotenoid) **violaxanthin**. Synthesis of violaxanthin is catalyzed by zeaxanthin epoxidase (ZEP), the enzyme encoded by the *ABA1* locus of *Arabidopsis*. This discovery provided conclusive evidence that ABA synthesis occurs via the "indirect" or carotenoid pathway, rather than as a small molecule. Maize mutants (*vp*) that are blocked at other steps in the carotenoid pathway also have reduced levels of ABA and exhibit **vivipary**—the precocious germination of seeds in the fruit while still attached to the plant. Vivipary is a feature of many ABA-deficient seeds.

Violaxanthin is converted to the C40 compound **9'-*cis*neoxanthin**, which is then cleaved to form the C15 compound **xanthoxal**, previously called *xanthoxin*, a neutral growth inhibitor that has physiological properties similar to those of ABA. The cleavage is catalyzed by **9-*cis*-**

epoxycarotenoid dioxygenase (**NCED**), so named because it can cleave both 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin. Synthesis of NCED is rapidly induced by water stress, suggesting that the reaction it catalyzes is a key regulatory step for ABA synthesis. The enzyme is localized on the thylakoids, where the carotenoid substrate is located. Finally, xanthoxal is converted to ABA via oxidative steps involving the intermediate(s) **ABA-aldehyde** and/or possibly xanthoxic acid. This final step is catalyzed by a family of aldehyde oxidases that all require a molybdenum cofactor; the *aba3* mutants of *Arabidopsis* lack a functional molybdenum cofactor and are therefore unable to synthesize ABA.

ABA Concentrations in Tissues Are Highly Variable

ABA biosynthesis and concentrations can fluctuate dramatically in specific tissues during development or in response to changing environmental conditions. In developing seeds, for example, ABAlevels can increase 100-fold within a few days and then decline to vanishingly low levels as maturation proceeds. Under conditions of water stress, ABA in the leaves can increase 50-fold within 4 to 8 hours. Upon rewatering, the ABAlevel declines to normal in the same amount of time. Biosynthesis is not the only factor that regulates ABA concentrations in the tissue. As with other plant hormones, the concentration of free ABAin the cytosol is also regulated by degradation, compartmentation, conjugation, and transport. For example, cytosolic ABA increases during water stress as a result of synthesis in the leaf, redistribution within the mesophyll cell, import from the roots, and recirculation from other leaves. The concentration of ABA declines after rewatering because of degradation and export from the leaf, as well as a decrease in the rate of synthesis.

ABA Can Be Inactivated by Oxidation or Conjugation

A major cause of the inactivation of free ABA is oxidation, yielding the unstable intermediate 6-hydroxymethyl ABA, which is rapidly converted to **phaseic acid** (**PA**) and **dihydrophaseic acid** (**DPA**). PAis usually inactive, or it exhibits greatly reduced activity, in bioassays. However, PAcan induce stomatal closure in some species, and it is as active as ABA in inhibiting gibberellic acid–induced �-amylase production in barley aleurone layers. These effects suggest that PA may be able to bind to ABA receptors. In contrast to PA, DPA has no detectable activity in any of the bioassays tested.

Free ABA is also inactivated by covalent conjugation to another molecule, such as a monosaccharide. A common example of an ABA conjugate is **ABA-b-D-glucosyl ester** (**ABA-GE**). Conjugation not only renders ABA inactive as a hormone; it also alters its polarity and cellular distribution. Whereas free ABAis localized in the cytosol, ABA-GE accumulates in vacuoles and thus could theoretically serve as a storage form of the hormone. Esterase enzymes in plant cells could release free ABA from the conjugated form. However, there is no evidence that ABA-GE hydrolysis contributes to the rapid increase in ABA in the leaf during water stress. When plants were subjected to a series of stress and rewatering cycles, the ABA GE concentration increased steadily, suggesting that the conjugated form is not broken down during water stress.

ABA Is Translocated in Vascular Tissue

ABA is transported by both the xylem and the phloem, but it is normally much more abundant in the phloem sap. When radioactive ABAis applied to a leaf, it is transported both up the stem and down toward the roots. Most of the radioactive ABA is found in the roots within 24 hours. Destruction of the phloem by a stem girdle prevents ABA accumulation in the roots, indicating that the hormone is transported in the phloem sap.

ABAsynthesized in the roots can also be transported to the shoot via the xylem. Whereas the concentration of ABA in the xylem sap of well-watered sunflower plants is between 1.0 and 15.0 n*M*, the ABAconcentration in waterstressed sunflower plants increases to as much as 3000 n*M* (3.0 μ*M*). The magnitude of the stress induced change in xylem ABA content varies widely among species, and it has been suggested that ABA also is transported in a conjugated form, then released by hydrolysis in leaves. However, the postulated hydrolases have yet to be identified.

As water stress begins, some of the ABA carried by the xylem stream is synthesized in roots that are in direct contact with the drying soil. Because this transport can occur before the low water potential of the soil causes any measurable change in the water status of the leaves, ABA is believed to be a root signal that helps reduce the transpiration rate by closing stomata in leaves.

Although a concentration of 3.0 μ*M* ABA in the apoplast is sufficient to close stomata, not all of the ABA in the xylem stream reaches the guard cells. Much of the ABA in the transpiration stream is taken up and metabolized by the mesophyll cells. During the early stages of water stress, however, the pH of the xylem sap becomes more alkaline, increasing from about pH 6.3 to about pH 7.2.

The major control of ABA distribution among plant cell compartments follows the "**anion trap"** concept: The dissociated (anion) form of this weak acid accumulates in alkaline compartments and may be redistributed according to the steepness of the pH gradients across membranes. In addition to partitioning according to the relative pH of compartments, specific uptake carriers contribute to maintaining a low apoplastic ABA concentration in unstressed plants.

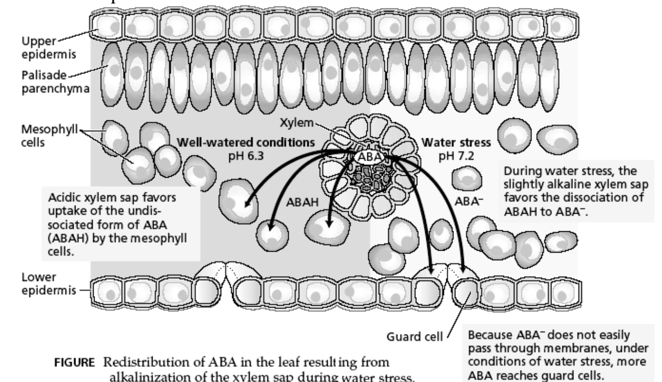


FIGURE Redistribution of ABA in the leaf resulting from alkalinization of the xylem sap during water stress.

Stress-induced alkalinization of the apoplast favors formation of the dissociated form of abscisic acid, ABA–, which does not readily cross membranes. Hence, less ABA enters the mesophyll cells, and more reaches the guard cells via the transpiration stream. Note that ABA is redistributed in the leaf in this way without any increase in the total ABA level. This increase in xylem sap pH may function as a root signal that promotes early closure of the stomata.

DEVELOPMENTAL AND PHYSIOLOGICAL EFFECTS OF ABA

Abscisic acid plays primary regulatory roles in the initiation and maintenance of seed and bud dormancy and in the plant's response to stress, particularly water stress. In addition, ABA influences many other aspects of plant development by interacting, usually as an antagonist, with auxin, cytokinin, gibberellin, ethylene, and brassinosteroids. In this section we will explore the diverse physiological effects of ABA, beginning with its role in seed development.

ABA Levels in Seeds Peak during Embryogenesis

Seed development can be divided into three phases of approximately equal duration:
1. During the first phase, which is characterized by cell divisions and tissue differentiation, the zygote undergoes embryogenesis and the endosperm tissue proliferates.
2. During the second phase, cell divisions cease and storage compounds accumulate.
3. In the final phase, the embryo becomes tolerant to desiccation, and the seed dehydrates, losing up to 90% of its water. As a consequence of dehydration, metabolism comes to a halt and the seed enters a **quiescent** ("resting") state. In contrast to dormant seeds, quiescent seeds will germinate upon rehydration.

The latter two phases result in the production of viable seeds with adequate resources to support germination and the capacity to wait weeks to years before resuming growth. Typically, the ABA content of seeds is very low early in embryogenesis, reaches a maximum at about the halfway point, and then gradually falls to low levels as the seed reaches maturity. Thus there is a broad peak of ABA accumulation in the seed corresponding to mid- to late embryogenesis.

The hormonal balance of seeds is complicated by the fact that not all the tissues have the same genotype. The seed coat is derived from maternal tissues; the zygote and endosperm are derived from both parents.

Genetic studies with ABA-deficient mutants of *Arabidopsis* have shown that the zygotic genotype controls ABA synthesis in the embryo and endosperm and is essential to dormancy induction, whereas the maternal genotype controls the major, early peak of ABA accumulation and helps suppress vivipary in mid-embryogenesis.

ABA Promotes Desiccation Tolerance in the Embryo

An important function of ABAin the developing seed is to promote the acquisition of desiccation tolerance. Desiccation can severely damage membranes and other cellular constituents. During the mid- to late stages of seed development, specific mRNAs accumulate in embryos at the time of high levels of endogenous ABA. These mRNAs encode so-called **late-embryogenesis-abundant** (**LEA**) proteins thought to be involved in desiccation tolerance. Synthesis of many LEA proteins, or related family members, can be induced by ABA treatment of either young embryos or vegetative tissues. Thus the synthesis of most LEA proteins is under ABA control.

ABA Promotes the Accumulation of Seed Storage Protein during Embryogenesis

Storage compounds accumulate during mid- to late embryogenesis. Because ABA levels are still high, ABA could be affecting the translocation of sugars and amino acids, the synthesis of the reserve materials, or both. Studies in mutants impaired in both ABA synthesis and response showed no effect of ABA on sugar translocation. In contrast, ABA has been shown to affect the amounts and composition of storage proteins. For example, exogenous ABA promotes accumulation of storage proteins in cultured embryos of many species, and some ABA-deficient or -insensitive mutants have reduced storage protein accumulation. However, storage protein synthesis is also reduced in other seed developmental mutants with normal ABA levels and responses, indicating that ABA is only one of several signals controlling the expression of storage protein genes during embryogenesis. ABA not only regulates the accumulation of storage proteins during embryogenesis; it can also maintain the mature embryo in a dormant state until the environmental conditions are optimal for growth. Seed dormancy is an important factor in the adaptation of plants to unfavorable environments. As we will discuss in the next few sections, plants have evolved a variety of mechanisms, some of them involving ABA, that enable them to maintain their seeds in a dormant state.

Seed Dormancy May Be Imposed by the Coat or the Embryo

During seed maturation, the embryo enters a quiescent phase in response to desiccation. Seed germination can be defined as the resumption of growth of the embryo of the mature seed; it depends on the same environmental conditions as vegetative growth does. Water and oxygen must be available, the temperature must be suitable, and there must be no inhibitory substances present.

In many cases a viable (living) seed will not germinate even if all the necessary environmental conditions for growth are satisfied. This phenomenon is termed **seed dormancy**. Seed dormancy introduces a temporal delay in the germination process that provides additional time for seed dispersal over greater geographic distances. It also maximizes seedling survival by preventing germination under unfavorable conditions. Two types of seed dormancy have been recognized: coat-imposed dormancy and embryo dormancy.

Coat-imposed dormancy. Dormancy imposed on the embryo by the seed coat and other enclosing tissues, such as endosperm, pericarp, or extrafloral organs, is known as **coat-imposed dormancy**. The embryos of such seeds will germinate readily in the presence of water and oxygen once the seed coat and other surrounding tissues have been either removed or damaged. There are five basic mechanisms of coat-imposed dormancy:

1. *Prevention of water uptake*.
2. *Mechanical constraint*. The first visible sign of germination is typically the radicle breaking through the seed coat. In some cases, however, the seed coat may be too rigid for the radicle to penetrate. For the seeds to germinate, the endosperm cell walls must be weakened by the production of cell wall–degrading enzymes.
3. *Interference with gas exchange*. Lowered permeability of seed coats to oxygen suggests that the seed coat inhibits germination by limiting the oxygen supply to the embryo.
4. *Retention of inhibitors*. The seed coat may prevent the escape of inhibitors from the seed.
5. *Inhibitor production*. Seed coats and pericarps may contain relatively high concentrations of growth inhibitors, including ABA, that can suppress germination of the embryo

Embryo dormancy. The second type of seed dormancy is **embryo dormancy**, a dormancy that is intrinsic to the embryo and is not due to any influence of the seed coat or other surrounding tissues. In some cases, embryo dormancy can be relieved by amputation of the cotyledons. Species in which the cotyledons exert an inhibitory effect include European hazel (*Corylus avellana*) and European ash (*Fraxinus excelsior*).

A fascinating demonstration of the cotyledon's ability to inhibit growth is found in species (e.g., peach) in which the isolated dormant embryos germinate but grow extremely slowly to form a dwarf plant. If the cotyledons are removed at an early stage of development, however, the plant abruptly shifts to normal growth.

Embryo dormancy is thought to be due to the presence of inhibitors, especially ABA, as well as the absence of growth promoters, such as GA (gibberellic acid). The loss of embryo dormancy is often associated with a sharp drop in the ratio of ABA to GA.

Primary versus secondary seed dormancy. Different types of seed dormancy also can be distinguished on the basis of the timing of dormancy onset rather than the cause of dormancy:

- Seeds that are released from the plant in a dormant state are said to exhibit **primary dormancy**.
- Seeds that are released from the plant in a non dormant state, but that become dormant if the conditions for germination are unfavorable, exhibit **secondary dormancy**.

For example, seeds of *Avena sativa* (oat) can become dormant in the presence of temperatures higher than the maximum for germination, whereas seeds of *Phacelia dubia* (small-flower scorpion weed) become dormant at temperatures below the minimum for germination. The mechanisms of secondary dormancy are poorly understood.

Environmental Factors Control the Release from Seed Dormancy

Various external factors release the seed from embryo dormancy, and dormant seeds typically respond to more than one of three factors:

1. *After ripening*. Many seeds lose their dormancy when their moisture content is reduced to a certain level by drying—a phenomenon known as **afterripening**.
2. *Chilling*. Low temperature, or **chilling**, can release seeds from dormancy. Many seeds require a period of cold (0–10°C) while in a fully hydrated (imbibed) state in order to germinate.
3. *Light*. Many seeds have a light requirement for germination, which may involve only a brief exposure, as in the case of lettuce, an intermittent treatment (e.g., succulents of the genus *Kalanchoe*), or even a specific photoperiod involving short or long days.

Seed Dormancy Is Controlled by the Ratio of ABA to GA

Mature seeds may be either dormant or nondormant, depending on the species. Nondormant seeds, such as pea, will germinate readily if provided with water only. Dormant seeds, on the other hand, fail to germinate in the presence of water, and instead require some additional treatment or condition. As we have seen, dormancy may arise from the rigidity or impermeability of the seed coat (coat imposed dormancy) or from the persistence of the state of arrested development of the embryo. Examples of the latter include seeds that require afterripening, chilling, or light to germinate.

ABA mutants have been extremely useful in demonstrating the role of ABA in seed dormancy. Dormancy of *Arabidopsis* seeds can be overcome with a period of after ripening and/or cold treatment. ABA-deficient (*aba*) mutants of *Arabidopsis* have been shown to be non-dormant at maturity. When reciprocal crosses between *aba* and wild type plants were carried out, the seeds exhibited dormancy only when the embryo itself produced the ABA. Neither maternal nor exogenously applied ABA was effective in inducing dormancy in an *aba* embryo. On the other hand, maternally derived ABA constitutes the major peak present in seeds and is required for other aspects of seed development—for example, helping suppress vivipary in midembryogenesis. Thus the two sources of ABA function in different developmental pathways. Dormancy is also greatly reduced in seeds from the ABA insensitive mutants *abi1* (*ABA-insensitive1*), *abi2*, and *abi3*, even though these seeds contain higher ABA concentrations than those of the wild type throughout development, possibly reflecting feedback regulation of ABA metabolism.

ABA-deficient tomato mutants seem to function in the same way, indicating that the phenomenon is probably a general one. However, other mutants with reduced dormancy, but normal ABA levels and sensitivity, point to additional regulators of dormancy. Although the role of ABA in initiating and maintaining seed dormancy is well established, other hormones contribute to the overall effect. For example, in most plants the peak of ABA production in the seed coincides with a decline in the levels of IAA and GA.

An elegant demonstration of the importance of the ratio of ABA to GA in seeds was provided by the genetic screen that led to isolation of the first ABA-deficient mutants of *Arabidopsis*. Seeds of a GA-deficient mutant that could not germinate in the absence of exogenous GA were mutagenized and then grown in the greenhouse.

The seeds produced by these mutagenized plants were then screened for **revertants**—that is, seeds that had regained their ability to germinate. Revertants were isolated, and they turned out to be mutants of abscisic acid synthesis. The revertants germinated because dormancy had not been induced, so subsequent synthesis of GA was no longer required to overcome it. This study elegantly illustrates the general principle that the balance of plant hormones is often more critical than are their absolute concentrations in regulating development. However, ABA and GA exert their effects on seed dormancy at different times, so their antagonistic effects on dormancy do not necessarily reflect a direct interaction.

Recent genetic screens for suppressors of ABA insensitivity have identified additional antagonistic interactions between ABA and ethylene or brassinosteroid effects on germination. In addition, many new alleles of ABA-deficient or *ABA-insensitive4* (*abi4*) mutants have been identified in screens for altered sensitivity to sugar. These studies show that a complex regulatory web integrates hormonal and nutrient signaling.

ABA Inhibits Precocious Germination and Vivipary

When immature embryos are removed from their seeds and placed in culture midway through development before the onset of dormancy, they germinate precociously—that is, without passing through the normal quiescent and/or

dormant stage of development. ABA added to the culture medium inhibits precocious germination. This result, in combination with the fact that the level of endogenous ABA is high during mid- to late seed development, suggests that ABA is the natural constraint that keeps developing embryos in their embryogenic state.

Further evidence for the role of ABA in preventing precocious germination has been provided by genetic studies of vivipary. The tendency toward vivipary, also known as *preharvest sprouting*, is a varietal characteristic in grain crops that is favored by wet weather. In maize, several viviparous (*vp*) mutants have been selected in which the embryos germinate directly on the cob while still attached to the plant.

Several of these mutants are ABA deficient (*vp2*, *vp5*, *vp7*, and *vp14*); one is ABA insensitive (*vp1*). Vivipary in the ABA-deficient mutants can be partially prevented by treatment with exogenous ABA. Vivipary in maize also requires synthesis of GA early in embryogenesis as a positive signal; double mutants deficient in both GAand ABA do not exhibit vivipary.

In contrast to the maize mutants, single-gene mutants of *Arabidopsis* (*aba1, aba3*, *abi1*, and *abi3*) fail to exhibit vivipary, although they are nondormant. The lack of vivipary might reflect a lack of moisture because such seeds will germinate within the fruits under conditions of high relative humidity. However, other *Arabidopsis* mutants with a normal ABA response and only moderately reduced ABA levels (e.g., *fusca3*, which belongs to a class of mutants1 defective in regulating the transition from embryogenesis to germination) exhibit some vivipary even at low humidities. Furthermore, double mutants combining either defects in ABA biosynthesis or ABA response with the *fusca3* mutation have a high frequency of vivipary, suggesting that redundant control mechanisms suppress vivipary in *Arabidopsis*.

ABA Accumulates in Dormant Buds

In woody species, dormancy is an important adaptive feature in cold climates. When a tree is exposed to very low temperatures in winter, it protects its meristems with bud scales and temporarily stops bud growth. This response to low temperatures requires a sensory mechanism that detects the environmental changes (sensory signals), and a control system that transduces the sensory signals and triggers the developmental processes leading to bud dormancy. ABA was originally suggested as the dormancy-inducing hormone because it accumulates in dormant buds and decreases after the tissue is exposed to low temperatures. However, later studies showed that the ABA content of buds does not always correlate with the degree of dormancy.

As we saw in the case of seed dormancy, this apparent discrepancy could reflect interactions between ABA and other hormones as part of a process in which bud dormancy and growth are regulated by the balance between bud growth inhibitors, such as ABA, and growth-inducing substances, such as cytokinins and gibberellins.

Although much progress has been achieved in elucidating the role of ABA in seed dormancy by the use of ABA-deficient mutants, progress on the role of ABAin bud dormancy, which applies mainly to woody perennials, has lagged because of the lack of a convenient genetic system. This discrepancy illustrates the tremendous contribution that genetics and molecular biology have made to plant physiology, and it underscores the need for extending such approaches to woody species.

Analyses of traits such as dormancy are complicated by the fact that they are often controlled by the combined action of several genes, resulting in a gradation of phenotypes referred to as *quantitative traits*. Recent genetic mapping studies suggest that homologs of *ABI1* may regulate bud dormancy in poplar trees. For a description of such studies

ABA Inhibits GA-Induced Enzyme Production

ABA inhibits the synthesis of hydrolytic enzymes that are essential for the breakdown of storage reserves in seeds. For example, GA stimulates the aleurone layer of cereal grains to produce α-amylase and other hydrolytic enzymes that break down stored resources in the endosperm during germination. ABAinhibits this GA-dependent enzyme synthesis by inhibiting the transcription of α- amylase mRNA. ABA exerts this inhibitory effect via at least two mechanisms:

1. VP1, a protein originally identified as an activator of ABA-induced gene expression, acts as a transcriptional repressor of some GA-regulated genes.
2. ABA represses the GA-induced expression of GAMYB, a transcription factor that mediates the GA induction of α-amylase expression.

ABA Closes Stomata in Response to Water Stress

Elucidation of the roles of ABA in freezing, salt, and water stress led to the characterization of ABA as a stress hormone. As noted earlier, ABA concentrations in leaves can increase up to 50 times under drought conditions—the most dramatic change in concentration reported for any hormone in response to an environmental signal. Redistribution or biosynthesis of ABA is very effective in causing stomatal closure, and its accumulation in stressed leaves plays an important role in the reduction of water loss by transpiration under water stress conditions.

Stomatal closing can also be caused by ABA synthesized in the roots and exported to the shoot. Mutants that lack the ability to produce ABA exhibit permanent wilting and are called *wilty* mutants because of their inability to close their stomata. Application of exogenous ABA to such mutants causes stomatal closure and a restoration of turgor pressure.

ABA Promotes Root Growth and Inhibits Shoot Growth at Low Water Potentials

ABA has different effects on the growth of roots and shoots, and the effects are strongly dependent on the water status of the plant. Endogenous ABA promotes shoot growth in well-watered plants by suppressing ethylene production. When water is limiting (i.e., at low water potentials), the opposite occurs: Shoot growth is greater in the ABA-deficient mutant than in the wild type. Thus, endogenous ABA acts as a signal to reduce shoot growth only under water stress conditions.

Now let's examine how ABA affects roots. When water is abundant, root growth is slightly greater in the wild type (normal endogenous ABA) than in the ABA-deficient mutant, similar to growth in shoots. Therefore, at high water potentials (when the total ABA levels are low), endogenous ABA exerts a slight positive effect on the growth of both roots and shoots.

Under dehydrating conditions, however, the growth of the roots is much higher in the wild type than in the ABA

deficient mutant, although growth is still inhibited relative to root growth of either genotype when water is abundant. In this case, endogenous ABA promotes root growth, apparently by inhibiting ethylene production during water stress.

To summarize, under dehydrating conditons, when ABA levels are high, the endogenous hormone exerts a strong positive effect on root growth by suppressing ethylene production, and a slight negative effect on shoot growth. The overall effect is a dramatic increase in the root:shoot ratio at low water potentials, which, along with the effect of ABA on stomatal closure, helps the plant cope with water stress. For another example of the role of ABA in the response to dehydration

ABA Promotes Leaf Senescence Independently of Ethylene

Abscisic acid was originally isolated as an abscission-causing factor. However, it has since become evident that ABA stimulates abscission of organs in only a few species and that the primary hormone causing abscission is ethylene. On the other hand, ABA is clearly involved in leaf senescence, and through its promotion of senescence it might indirectly increase ethylene formation and stimulate abscission.

Leaf segments senesce faster in darkness than in light, and they turn yellow as a result of chlorophyll breakdown. In addition, the breakdown of proteins and nucleic acids is increased by the stimulation of several hydrolases. ABA greatly accelerates the senescence of both leaf segments and attached leaves.

CELLULAR AND MOLECULAR MODES OF ABA ACTION

ABA is involved in short-term physiological effects (e.g., stomatal closure), as well as long-term developmental processes (e.g., seed maturation). Rapid physiological responses frequently involve alterations in the fluxes of ions across membranes and may involve some gene regulation as well, and long-term processes inevitably involve major changes in the pattern of gene expression. Signal transduction pathways, which amplify the primary signal generated when the hormone binds to its receptor, are required for both the short-term and the long term effects of ABA. Genetic studies have shown that many conserved signaling components regulate both short- and long-term responses, indicating that they share common signaling mechanisms. In this section we will describe what is known about the mechanism of ABA action at the cellular and molecular levels.

ABA Is Perceived Both Extracellularly and Intracellularly

Although ABA has been shown to interact directly with phospholipids, it is widely assumed that the ABA receptor is a protein. To date, however, the protein receptor for ABA has not been identified. Experiments have been performed to determine whether the hormone must enter the cell to be effective, or whether it can act externally by binding to a receptor located on the outer surface of the plasma membrane. The results so far suggest multiple sites of perception. Some experiments point to a receptor on the outer surface of the cell. For example, microinjected ABA fails to alter stomatal opening in the spiderwort *Commelina*, or to inhibit GA-induced α-amylase synthesis in barley aleurone protoplasts. Furthermore, impermeant ABA–protein conjugates have been shown to activate both ion channel activity and gene expression.

Other experiments, however, support an intracellular location for the ABA receptor: Extracellular application of ABA was nearly twice as effective at inhibiting stomatal opening at pH 6.15, when it is fully protonated and readily taken up by guard cells, versus at pH 8, when it is largely dissociated to the anionic form that does not readily cross membranes.

- ABA supplied directly and continuously to the cytosol via a patch pipette inhibited K+ in channels, which are required for stomatal opening .
- Microinjection of an inactive "caged" form of ABA into guard cells of *Commelina* resulted in stomatal closure after the stomata were treated briefly with UV irradiation to activate the hormone—that is, release it from its molecular cage. Control guard cells injected with a nonphotolyzable form of the caged ABA did not close after UV irradiation.

Taken together, these results indicate that extracellular perception of ABA can prevent stomatal opening and regulate gene expression, and intracellular ABA can both induce stomatal closure and inhibit the K+ in current required for opening. Thus there appear to be both extracellular and intracellular ABA receptors. However, they have yet to be identified or localized.

ABA Increases Cytosolic Ca^{2+}, Raises Cytosolic pH, and Depolarizes the Membrane

Stomatal closure is driven by a reduction in guard cell turgor pressure caused by a massive long-term efflux of K+ and anions from the cell. During the subsequent shrinkage of the cell due to water loss, the surface area of the plasma membrane may contract by as much as 50%. Where does the extra membrane go? The answer seems to be that it is taken up as small vesicles by endocytosis—a process that also involves reorganization of the actin cytoskeleton. However, the first changes detected after exposure of guard cells to ABA are transient membrane depolarization caused by the net influx of positive charge, and transient increases in the cytosolic calcium concentration.

ABA stimulates elevations in the concentration of cytosolic Ca2+ by inducing both influx through plasma membrane channels and release of calcium into the cytosol from internal compartments, such as the central vacuole. Stimulation of influx occurs via a pathway that uses **reactive oxygen species** (**ROS**), such as hydrogen peroxide (H_2O_2) or superoxide (O^{2-}), as secondary messengers leading to plasma membrane channel activation. Calcium release from intracellular stores can be induced by a variety of second messengers, including inositol 1,4,5- trisphosphate (IP3), cyclic ADP-ribose (cADPR), and self- amplifying (calcium-induced) Ca^{2+} release. Recent studies have shown that ABA stimulates **nitric oxide** (**NO**) synthesis in guard cells, which induces stomatal closure in a cADPR-dependent manner, indicating that NO is an even earlier secondary messenger in this response pathway.

The combination of calcium influx and the release of calcium from internal stores raises the cytosolic calcium concentration from 50 to 350 n*M* to as high as 1100 n*M* (1.1 m*M*). This increase is sufficient to cause stomatal closure, as demonstrated by the following experiment.

As in the experiment described earlier, calcium was microinjected into guard cells in a caged form that could be hydrolyzed by a pulse of UV light. This method allowed the

investigators to control both the concentration of free calcium and the time of release to the cytosol. At cytosolic concentrations of 600 n*M* or more, release of calcium from its cage triggered stomatal closure. This level of intracellular calcium is well within the concentration range observed after ABA treatment.

In the preceding studies, intracellular free calcium was measured by the use of microinjected calcium-sensitive ratiometric fluorescent dyes2, such as fura-2 or indo-1. However, microinjections of fluorescent dyes into single plant cells are difficult and often result in cell death. Success rates of viable injections into *Arabidopsis* guard cells can be less than 3%. In contrast, transgenic plants expressing the gene for the calcium indicator protein **yellow cameleon** make it possible to monitor several fluorescing cells in parallel, without the need for invasive injections. Such studies have demonstrated that the cytosolic Ca^{2+} concentration oscillates with distinct periodicities, depending on the signals received.

These results support the hypothesis that an increase in cytosolic calcium, partly derived from intracellular stores, is responsible for ABA-induced stomatal closure. However, the growth hormone auxin can induce stomatal opening, and this auxin-induced stomatal opening, like ABA-induced stomatal closure, is accompanied by *increases* in cytosolic calcium. This finding suggests that the detailed characteristics of the location and periodicity of Ca^{2+} oscillations (the "**Ca^{2+} signature"),** rather than the overall concentration of cytosolic calcium, determine the cellular response. In addition to increasing the cytosolic calcium concentration, ABA causes an alkalinization of the cytosol from about pH 7.67 to pH 7.94. The increase in cytosolic pH has been shown to activate the K+ efflux channels on the plasma membrane apparently by increasing the number of channels available for activation.

ABA Activation of Slow Anion Channels Causes Long-Term Membrane Depolarization

The rapid, transient depolarizations induced by ABA are insufficient to open the K+ efflux channels, which require long-term membrane depolarization in order to open. However, long-term depolarizations in response to ABA have been demonstrated. According to a widely accepted model, long-term membrane depolarization is triggered by two factors: (1) an ABA-induced transient depolarization of the plasma membrane, coupled with (2) an increase in cytosolic calcium. Both of these conditions are required to open calcium-activated slow (S-type) anion channels on the plasma membrane. ABAhas been shown to activate slow anion channels in guard cells.

The prolonged opening of these slow anion channels permits large quantities of Cl– and malate2– ions to escape from the cell, moving down their electrochemical gradients. (The inside of the cell is negatively charged, thus pushing Cl^- and $malate^{2-}$ out of the cell, and the outside has lower Cl^- and $malate^{2-}$ concentrations than the interior.) The outward flow of negatively charged Cl^- and $malate^{2-}$ ions generated in this way strongly depolarizes the membrane, triggering the voltage-gated K+ efflux channels to open.

In support of this model, inhibitors that block slow anion channels, such as 5-nitro-2,3-phenylpropylaminobenzoic acid (NPPB), also block ABA-induced stomatal closing. Inhibitors of the rapid (R-type) anion channels, such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), have no effect on ABA-induced stomatal closing .

Another factor that can contribute to membrane depolarization is inhibition of the plasma membrane H+-ATPase. ABAinhibits blue light–stimulated proton pumping by guard cell protoplasts, consistent with the model that the depolarization of the plasma membrane by ABA is partially caused by a decrease in the activity of the plasma membrane H+-ATPase. However, ABAdoes not inhibit the proton pump directly.

In *Vicia faba* (broad bean), at least, the plasma membrane H+-ATPase of the leaves is strongly inhibited by calcium. A calcium concentration of 0.3 µ*M*blocks 50% of the activity of H+-ATPase, and 1 µ*M* calcium blocks the enzyme completely. It appears that two factors contribute to ABAinhibition of the plasma membrane proton pump: an increase in the cytosolic Ca^{2+} concentration, and alkalinization of the cytosol.

In addition to causing stomatal closure, ABA prevents light-induced stomatal opening. In this case ABA acts by inhibiting the inward K^+ channels, which are open when the membrane is hyperpolarized by the proton pump. Inhibition of the inward K+ channels is mediated by the ABA-induced increase in cytosolic calcium concentration. Thus calcium and pH affect guard cell plasma membrane channels in two ways:

1. They prevent stomatal opening by inhibiting inward K^+ channels and plasma membrane proton pumps.
2. They promote stomatal closing by activating outward anion channels, thus leading to activation of K^+ efflux channels.

ABA Stimulates Phospholipid Metabolism

As discussed previously, much evidence supports a role for calcium both in the promotion of stomatal closing and in the inhibition of stomatal opening. According to the classic calcium-dependent signal transduction pathway of animal cells, IP_3 is released, along with diacylglycerol (DAG), when phospholipase C is activated by a G-protein in the plasma membrane . Does ABAuse the same pathway when it induces stomatal closure? In agreement with this model, ABA has been shown to stimulate phosphoinositide metabolism in *Vicia faba* (broad bean) guard cells. To detect the effect of ABA on IP3 release, it was necessary to include Li+ in the incubation medium as an inhibitor of inositol phosphatase, which rapidly removes phosphate groups from IP3. Under these conditions, a 90% ABA-induced increase in the level of IP3 was measured within 10 seconds of hormone treatment. Recent studies in *Arabidopsis* using antisense DNA to block expression of an ABAinduced phospholipase C have shown that this enzyme is required for ABA effects on germination, growth, and gene expression.

Heterotrimeric G-proteins may mediate the effects of ABA on stomatal movements. For example, in *Vicia faba* most studies have shown that G-protein activators, such as GTPαS, can inhibit the activity of the inward K+ channels. Consistent with the inhibitor results, ABA failed to inhibit inward K+ channels or light-induced stomatal opening in an *Arabidopsis* mutant with a defective Gα subunit. However, ABA still promoted stomatal closure in this mutant, indicating that inhibition of opening and promotion of closing take two distinct paths to the same end point—that is, closed stomata.

Other potential second messengers mediating the ABA response, such as phosphatidic acid and *myo*-inositol-hexaphosphate (IP6) have been identified, but the relationship of these compounds to IP3 and Ca2+ signaling is not yet known. All of these experiments indicate that

stomatal guard cells respond to multiple signals, possibly involving multiple receptors and overlapping signal transduction pathways.

Protein Kinases and Phosphatases Participate in ABA Action

Nearly all biological signaling systems involve protein phosphorylation and dephosphorylation reactions at some step in the pathway. Thus we can expect that signal transduction in guard cells, with their multiple sensory inputs, involves protein kinases and phosphatases. Artificially raising the ATP concentration inside guard cells by allowing the cytoplasm to equilibrate with the solution inside a patch pipette strongly activates the slow anion channels.

This activation of the slow anion channels by ATP is abolished by the inclusion of protein kinase inhibitors in the patch pipette solution. Protein kinase inhibitors also block ABA-induced stomatal closing. In contrast, lowering the concentration of ATP in the cytosol inactivates the slow anion channels. Additional experiments confirm that this inactivation is due to the presence of protein phosphatases, which remove phosphate groups that are covalently attached to proteins. In view of these results, it appears that protein phosphorylation and dephosphorylation play important roles in the ABA signal transduction pathway in guard cells.

There is now direct evidence for an ABA-activated protein kinase (AAPK) in *Vicia faba* guard cells. AAPK activity appears to be required for ABA activation of S-type anion currents and stomatal closing. This enzyme is an autophosphorylating protein kinase that either forms part of a Ca2+-independent signal transduction pathway for ABA, or acts farther downstream of calcium-induced signaling events. (The presence of both Ca2+-dependent and Ca2+-independent pathways for ABA action will be discussed shortly.) In addition, two Ca2+-dependent protein kinases, as well as MAP kinases, have been implicated in the ABA regulation of stomatal aperture.

The analysis of ABA-insensitive mutants has begun to help in the identification of genes coding for components of the signal transduction pathway. The *Arabidopsis abi1-1* and *abi2-1* mutations result in insensitivity to ABA in both seeds and adult plants. These *abi* mutants display phenotypes consistent with a defect in ABA signaling, including reduced seed dormancy, a tendency to wilt (due to improper regulation of stomatal aperture), and decreased expression of various ABA-inducible genes.

The defects in stomatal response include the ABA insensitivity of S-type anion channels—both inward and outward K+ channels—and actin reorganization. Although non-responsive to ABA, the mutant stomata will close when exposed to high external concentrations of Ca2+, suggesting that they are defective in their ability to initiate Ca2+ signaling. Consistent with this finding, ABA does not induce Ca2+ oscillations in these mutants.

ABI Protein Phosphatases Are Negative Regulators of the ABA Response

The *Arabidopsis ABI1* and *ABI2* genes have been cloned and identified as encoding two closely related serine/threonine protein phosphatases. This finding suggests that ABI1 and ABI2 regulate the activity of target proteins by dephosphorylating specific serine or threonine residues, but none of their substrates have been definitively identified. Because the *abi1-1* and *abi2-1* mutations result in decreased response to ABA, it was initially assumed that the wild-type genes *promote* the ABA response. However, the original mutations turned out to be dominant rather than recessive, and recent studies have shown that they act as "dominant negatives"; that is, one defective copy of the gene is sufficient to disrupt the ABA response by poisoning the activity of the functional gene products from the remaining wild-type allele.

Subsequently, recessive mutants of *ABI1* were obtained that exhibited a simple loss of *ABI1* activity. These recessive mutants of *ABI1* actually showed increased ABA sensitivity. Furthermore, overproducing the wild-type gene products or their homologs (closely related proteins) by reintroducing the gene into plants, under control of a highly expressed promoter, confers *reduced* ABA sensitivity. Thus the wild-type function of these protein phosphatases is to inhibit the ABA response.

ABA Signaling Also Involves Ca^{2+}-Independent Pathways

Although an ABA-induced increase in cytosolic calcium concentration is a key feature of the current model for ABA-induced guard cell closure, ABA is able to induce stomatal closure even in guard cells that show no increase in cytosolic calcium. In other words, ABA seems to be able to act via one or more calcium-independent pathways.

In addition to calcium, ABA can utilize cytosolic pH as a signaling intermediate. As previously discussed, a rise in cytosolic pH can lead to the activation of outward K+ channels, and one effect of the *abi1* mutation is to render these K+ channels insensitive to pH.

Such redundancy in the signal transduction pathways explains how guard cells are able to integrate a wide range of hormonal and environmental stimuli that affect stomatal aperture, and such redundancy is probably not unique to guard cells.

A simplified general model for ABA action in stomatal guard cells is shown in Figure. For clarity, only the cell surface receptors are shown. (see next page)

ABA Regulation of Gene Expression Is Mediated by Transcription Factors

Downstream of the early ABA signal transduction processes already discussed, ABA causes changes in gene expression. ABA has been shown to regulate the expression of numerous genes during seed maturation and under certain stress conditions, such as heat shock, adaptation to low temperatures, and salt tolerance. The ABA and stress-induced genes are presumed to contribute to adaptive aspects of induced tolerance.

They include genes encoding proteases, chaperonins, proteins similar to LEA proteins, enzymes of sugar or other compatible solute3 metabolism, ion and water channel proteins, enzymes that detoxify active oxygen species, and regulatory proteins such as transcription factors and protein kinases.

In a few cases, stimulation of transcription by ABA has been demonstrated directly. Gene activation by ABA is mediated by transcription factors. Four main classes of regulatory

1. ABA binds to its receptors.

2. ABA-binding induces the formation of reactive oxygen species, which activate plasma membrane Ca^{2+} channels.

3. ABA increases the levels of cyclic ADP-ribose and IP_3, which activate additional calcium channels on the tonoplast.

4. The influx of calcium initiates intracellular calcium oscillations and promotes the further release of calcium from vacuoles.

5. The rise in intracellular calcium blocks K^{+}_{in} channels.

6. The rise in intracellular calcium promotes the opening if Cl^{-}_{out} (anion) channels on the plasma membrane, causing membrane depolarization.

7. The plasma membrane proton pump is inhibited by the ABA-induced increase in cytostolic calcium and a rise in intracellular pH, further depolarizing the membrane.

8. Membrane depolarization activates K^{+}_{out} channels.

9. K^+ and anions to be released across the plasma membrane are first released from vacuoles into the cytosol.

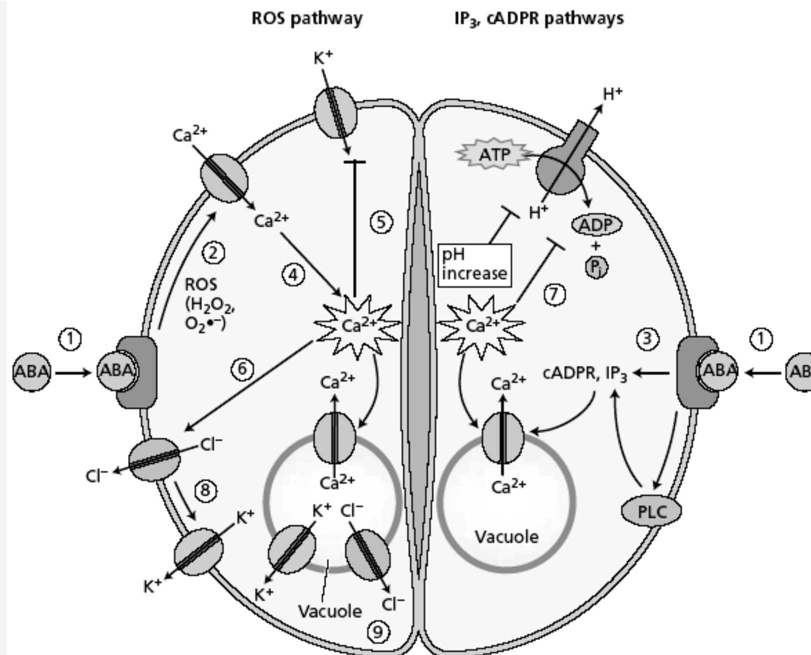


FIGURE Simplified model for ABA signaling in stomatal guard cells. The net effect is the loss of potassium and its anion (Cl^- or malate^{2-}) from the cell. (R = receptor; ROS = reactive oxygen species; cADPR = cyclic ADP-ribose; G-protein = GTP-binding protein; PLC = phospholipiase C.)

sequences conferring ABA inducibility have been identified, and proteins that bind to these sequences have been characterized. Under stress conditions, induction of gene expression may be ABA dependent or ABAindependent, and additional transcription factors have been identified that specifically mediate responses to cold, drought, or salt .

A few DNA elements have been identified that are involved in transcriptional repression by ABA. The best characterized of these are the gibberellin response elements (GAREs) that mediate the gibberellin-inducible, ABA repressible expression of the barley α-amylase gene .

Four transcription factors involved in ABA gene activation in maturing seeds have been identified by genetic means; mutations in the genes encoding any of these proteins reduce seed ABA responsiveness. The maize *VP1* (*VIVIPAROUS-1*) and *Arabidopsis ABI3* (*ABA-INSENSITIVE3*) genes encode highly similar proteins, and the *ABI4* and *ABI5* genes encode members of two other transcription factor families. *VP1/ABI3*, and *ABI4* are members of gene families found only in plants. In contrast, *ABI5* is a member of the basic leucine zipper (bZIP) family, whose members are present in all eukaryotes.

Additional members of the *ABI5* subfamily have been identified by nongenetic means and are also correlated with ABA-, embryonic-, drought-, or salt stress–induced gene expression. Characterization of *vp1*, *abi4*, and *abi5* mutants has shown that each of these genes can either activate or repress transcription, depending on the target gene. Because the promoter of any given gene contains binding sites for a variety of regulators, it is likely that these transcription factors act in complexes made up of varying combinations of regulators, whose composition is determined by the combination of available regulators and binding sites. To date, the protein ABI3/VP1 has been shown to interact physically with a variety of proteins, including ABI5 and its rice homolog (TRAB1). ABI5 also forms homodimers and heterodimers with other bZIP family members. There is additional evidence for indirect interactions that may be mediated by 14-3-3 proteins, a class of acidic proteins that dimerize and facilitate protein–protein interactions in a variety of signaling, transport, and enzymatic functions (**see** been characterized. Under stress conditions, induction of gene expression may be ABA dependent or ABAindependent, and additional transcription factors have been identified that specifically mediate responses to cold, drought, or salt.

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Other Negative Regulators of the ABA Response Have Been Identified

As described already, negative regulators of the ABA response (protein phosphatases) have been identified by isolation of dominant negative mutants such as *abi1* and *abi2* that result in ABA-insensitive phenotypes (analogous to the dominant negative effects of the ethylene receptor mutant *etr)*

Other negative regulators have been identified through isolation of mutants exhibiting enhanced responses to ABA. Mutants showing increased sensitivity to ABA during germination include *era* (*e*nhanced *r*esponse to *A*BA) and *abh* (*AB*A *h*ypersensitive). The *era* and *abh* mutants both confer ABA hypersensitivity in both stomatal closing and germination, making these mutants resistant to wilting and mildly drought tolerant.

Farnesyl transferase. The *ERA1* gene was cloned, and its protein product was identified as a subunit of the enzyme farnesyl transferase. Farnesyl transferases catalyze attachment of the isoprenoid intermediate farnesyl diphosphate to proteins that contain a specific signal sequence of amino acids. Many proteins that have been shown to participate in signal transduction are farnesylated. Farnesylated proteins are anchored to the membrane via hydrophobic interactions between the farnesyl group and the membrane lipids. The identification of ERA1 as part of farnesyl transferase suggests that a protein that normally suppresses the ABAresponse requires farnesylation and is possibly anchored to the membrane.

mRNAprocessing. *ABH1* encodes an mRNA 5′cap–binding protein that may be involved in mRNA processing of negative regulators of ABA signaling. (Recall that eukaryotic messenger RNAs have a "cap" consisting of methylated guanosine at the 5′end.) Comparison of transcript accumulation in wild-type and *abh1* plants showed a small number of misexpressed genes in the mutant, including some encoding possible signaling molecules.

Ethylene insensitivity. *ERA3* was found to be allelic to a previously identified ethylene signaling locus, *ETHYLENEINSENSITIVE 2* (*EIN2*). In addition to displaying defects in ABAand ethylene responses, mutations in this gene result in defects in the responses to auxin, jasmonic acid, and stress. This gene encodes a membrane-bound protein that appears to represent a point of "cross-talk"—i.e., a common signaling intermediate— mediating the responses to many different signals.

IP3 catabolism. Other screens have identified ABA signaling mutants on the basis of incorrect expression of reporter genes controlled by ABA-responsive promoters. Although the defects in some of these mutants are limited to gene expression, others affect plant growth responses. One such mutant, termed *fiery* (*fry*) to reflect the intensity of light emission by its ABA/stress-responsive luciferase reporter, is also hypersensitive to ABAand stress inhibition of germination and growth. The *FIERY* gene encodes an enzyme required for IP3 catabolism. The mutant phenotype demonstrates that the ability to attenuate, as well as induce, stress signaling is important for successful induction of stress tolerance.

Similar to the signaling mechanisms documented for other plant hormones, ABA signaling involves the coordinated action of positive and negative regulators affecting processes as diverse as transcription, RNA processing, protein phosphorylation or farnesylation, and metabolism of secondary messengers. As the signaling components are identified, and often are found to function in responses to multiple signals, the next challenge is to determine how they can lead to ABA-specific responses.

Other potential plant hormones

As it is so difficult to write a comprehensive definition of a plant hormone it is not surprising that many other plant compounds might fall within this group. In recent years many signaling molecules have been discovered which may act as hormones. For some molecules the evidence is good, with production, transport, a receptor and responses having been demonstrated. For other substances the evidence is less conclusive, although rapid progress is being made.

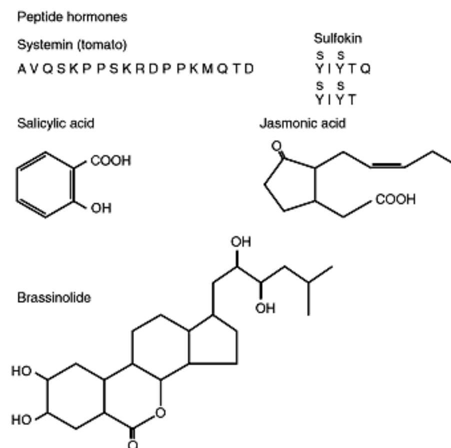


Fig: 4 Structures of some other plant hormones. The peptide hormones systemin and sulfokin (which is sulphated on the tyrosine residues) are synthesized from longer precursors. Both salicylic acid and jasmonic acid are involved in plant defence. Volatile derivatives can be produced in which the carboxyl group is methylated (–COOCH3). Brassinolide, a steroid lactone, is a brassinosteroid hormone.

Peptides

In animals numerous peptide hormones have been discovered, and it is surprising that so little is known about equivalent molecules in plants. However, there is good evidence that an 18-amino-acid peptide, systemin (Fig. 4), acts as a hormone in wounded leaves of tomato (*Lycopersicon esculentum*). This peptide is released from wounded cells and is transported through the phloem to unwounded leaves where it induces proteinase inhibitor (pin) gene expression. The proteinase inhibitors impair digestion in herbivores and hence have an antifeedant role. Systemin is active at very low (femtomolar) concentrations and a receptor has been isolated. Peptides closely related to systemin have been discovered in other members of the Solanaceae.

Another peptide, sulfokin, has been implicated in the control of cell division in asparagus (Asparagus officinalis) and rice cell cultures. This peptide is even smaller than systemin, being only 4 –5 amino acids long, two of which are sulphated (Fig. 4). Other biologically active peptides have been described.

However, in many cases, these peptides may act at the cell surface, performing an important role in signalling positional information. Whilst this is a fascinating area of plant biology, these substances cannot be considered plant growth hormones without evidence of transport within the plant. Nevertheless, there are undoubtedly many other peptide hormones in plants awaiting discovery.

RNA

Recently, small RNA molecules have been identified in the phloem which may act as transportable signals regulating gene expression. To date, these have been shown to be important in plant defence responses to viruses and in the silencing of synthetic genes introduced by genetic modification, both of which may be considered 'foreign' genes. This phenomenon is called post-transcriptional gene silencing (PTGS) and involves small double-stranded RNA molecules, known as short interfering RNAs (siRNAs), which are approximately 25 nucleotides long, and which mediate the destruction of specific target mRNAs. In addition, a number of groups have identified a separate class of microRNAs (miRNAs) which may regulate normal plant development. These are single-stranded RNAs of 21–22 nucleotides which are complementary to the genes encoding many regulatory proteins. Recently, the importance of miRNAs in leaf development has been demonstrated. As many hundreds of these miRNAs have been identified it seems possible that they may represent a more general means of regulating gene expression, and hence these small RNAs may be considered to be plant growth regulators.

Salicylic acid/jasmonic acid

These two compounds have been considered together as although they are structurally quite different they appear to interact closely to coordinate plant defence responses. Jasmonic acid (JA) is a 12 –carbon fatty acid derivative of linolenic acid whereas salicylic acid (SA) is a simple aromatic ring with a hydroxyl and carboxylic acid sidegroup (Fig. 4).

SA is involved in the expression of many pathogenesis-related (PR) proteins which have antifungal and antibacterial activities in response to pathogen attack. JA has been implicated in wound responses such as those caused by herbivores. It has been proposed that many SA-induced genes are repressed by JA and vice versa, and in this way a more 'directed' response is achieved without the diversion of resources into unnecessary protein production. Ethylene also plays a role in this coordinated gene expression. Some pathogens may exploit this interaction – the bacterium Pseudomonas syringae produces corotanine, an analogue of JA. This compound may repress the expression of the PR proteins, allowing infection to proceed. The complexity of signaling mechanisms in plant defence is beginning to be unravelled using a combination of mutants and gene expression profiling, allowing interactions between these pathways to be elucidated.

Both SA and JA can be methylated in vivo and are volatile in this form. In the laboratory it has been demonstrated that gene expression in neighbouring plants can be induced by these volatile compounds, although this remains to be convincingly demonstrated in field conditions. Plants contain many volatile compounds, a number of which have the potential to act as signalling molecules. Interestingly, this signalling may extend to organisms other than plants. Compounds released from herbivore-damaged plants have the potential to attract predators and parasites of the herbivores.

Brassinosteroids

More than 40 different brassinosteroid molecules have been found within plants (Fig. 4). These are all thought to be synthesized from the precursor campesterol. Brassinosteroids have been implicated in many roles including stem elongation, pollen tube growth, leaf bending and unrolling, stimulation of ethylene production, tracheary element differentiation and cell elongation. Plants with mutations in brassinosteroid metabolism are dwarf and exhibit unusual development in darkness. The transport of these molecules has not been well documented, but clearly they have a profound effect on plant development.

Other plant growth hormones will undoubtedly be found and, in time, the boundaries between the traditional hormones and the plethora of signalling molecules which exist within, and pass between, the cells of plants will become more blurred.

E. Sensory photobiology: Structure, function and mechanisms of action of phytochromes, cryptochromes and phototropins; photoperiodism and biological clocks. Stomatal Opening

Photomorphogenesis

1 Introduction

Light is critically important to plants. The majority of them are photosynthetic and light provides the energy source required for growth. However, light is equally important for the normal development of plants as an information medium. In the environment light is a very complex and dynamic signal. It varies in quantity, quality (colour) and direction over timescales ranging from seconds to months (Fig. 1). These different variables can indicate the passing of the seasons, the availability of new habitats for growth or the presence of neighbouring vegetation which may compete for resources. Therefore it is not surprising that many aspects of plant growth and development are strongly influenced by light. The plant, too, is a complicated and ever-changing system, and the response of a plant to a given set of environmental conditions will depend upon its developmental state. Plants pass through a juvenile state where their response to environmental signals differs from that of mature plants. Likewise, signals which stimulate a mature plant to flower may cause the seed of the same species to germinate – radically different developmental pathways. Similarly, plant responses are species-specific. Whilst a fast-growing weed such as Chenopodium album will respond to shaded conditions (i.e. low light) by elongating rapidly, rainforest tree seedlings can persist under a vegetation canopy for many years and commence rapid growth only when a gap opens in the forest canopy.

The complex nature of light as an environmental signal and the complex ways in which plants respond to it are reflected in the multitude of photoreceptors which have been identified. We are now beginning to understand how some of these photoreceptors work and interact with each other to coordinate plant development. Much of this research has, necessarily, been performed under highly artificial conditions allowing the roles of single (or at most, a few) photoreceptors to be studied. However, the information derived from these experiments is increasingly being applied to plants growing in a natural or agricultural environment.

2 The switch from etiolated to de-etiolated growth

A very simple, yet informative, experiment is to grow two sets of the same species of a plant in light and in darkness. The two sets of plants may then be scarcely recognizable as belonging to the same species. Whilst the light-grown individuals will be green, sturdy, with expanded leaves and ofmoderate height, the specimens grown in the dark will be etiolated – unpigmented, very tall, thin and weak with rudimentary or folded leaves. A plant which has been grown in darkness and then exposed to light will switch from etiolated to de-etiolated growth – the control of development by light being termed photomorphogenesis (photo = light, morpho = form, genesis = origin). Light regulates many aspects of plant development, inhibiting internode elongation, promoting leaf expansion (dicotyledons) or leaf unrolling (monocotyledons), promoting chlorophyll synthesis and chloroplast development and stimulating the synthesis of secondary products such as anthocyanin
pigments. These processes can be initiated at light energies far below that necessary for photosynthesis, in many cases by brief exposure only, and can occur in plants where the photosynthetic apparatus has not yet developed.

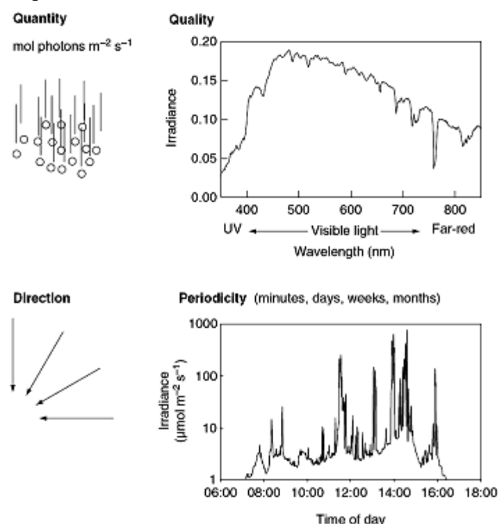


Fig: 1 Light is a complex and dynamic signal. Plants respond to many different aspects of the light environment. The quantity of light falling on a plant can be expressed in many different ways, but plant biologists generally refer to the number of photons falling per unit area per unit time. Light quality is also an important determinant of plant responses. Photosynthetically active radiation (PAR) is roughly similar to visible light and extends from 400 nm to 700 nm. Plants also respond to UV and far-red light. The light quality spectrum is that of full sunlight taken in Sheffield, UK. The variation in irradiance over the course of a day was measured in a tropical rainforest. Irradiance is very low most of the day (note the logarithmic scale) but increases as light intermittently penetrates gaps in the forest canopy. The direction at which light falls upon a plant and the way in which it varies over time is also important.

Light is perceived by a series of photoreceptors, the best studied of which are the phytochromes. However, plants contain several distinct families of photoreceptors in addition to phytochrome – these include the blue/UV-A photoreceptors (cryptochromes), poorly characterized UV-B photoreceptors and the phototropins (involved in phototropic responses).

3 Phytochrome and photomorphogenesis

3.1 The discovery of phytochrome

Our understanding of plant photoreceptors has been revolutionized in recent years by the use of molecular genetic techniques coupled with careful biochemical and physiological measurements. By isolating and characterizing the genes encoding the different

photoreceptors, and by producing mutant and transgenic plants with lesions in photoperception, it has been possible to build a framework describing some of the mechanisms by which plants respond to light. However, this current revolution is based upon a long, and distinguished, history of studying plant responses to light.

The existence of phytochrome was first deduced from studies of certain varieties of lettuce (*Lactuca sativa*) seeds which require light for germination. These seeds are induced to germinate by brief (a few minutes) exposure to red light of low irradiance, whilst a similar exposure to far-red light is inhibitory to germination. Careful measurements made at many different wavelengths allowed the action spectra of these two responses to be established (Fig. 2).

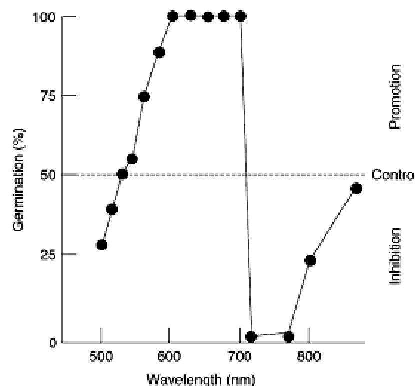


Fig: 2 The action spectrum of germination of Grand Rapids lettuce (Lactuca sativa). Approximately 50% of untreated, control seeds placed in darkness germinate. This increases to 100% when seeds are illuminated with red light, and falls close to zero if they are illuminated with far-red light.

The stimulation of germination was found to have a maximum in the red at 660 nm whilst the inhibition of germination by far-red light had a maximum at 730 nm. A key breakthrough came with the discovery that alternating exposure to red and far-red light can be given formany cycles and whichever wavelength is given last determines the response. These observations led Hendricks and Borthwick, working in Beltsville, Maryland in the 1950s, to postulate the existence of a photoreceptor named phytochrome, existing in two photointerconvertible forms. One form, termed PR, absorbs red light and is converted to the other form PFR. The spectral properties of PFR are such that it absorbs light with a peak in the far-red and under far-red illumination it is converted to PR again (Fig. 3). PFR also reverts slowly to PR when plants are placed in complete darkness. Many models of phytochrome action have proposed that PFR is the active form, whilst PR is inactive. However, as will become apparent later in this chapter, such a simple interpretation may not always be entirely accurate.

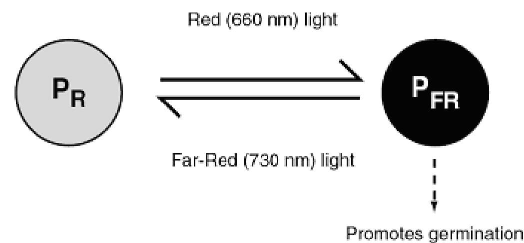


Fig: 3 Phytochrome exists in two photointer-convertible states. Phytochrome is synthesized in the dark in the PR form which predominantly absorbs red light. Exposure to red light results in conversion to the PFR form which triggers many phytochrome-regulated responses. The PFR form can be converted back into the PR form by exposure to far-red light.

Subsequently phytochrome was isolated from plant tissues. It is alow-abundance, blue-green chromoprotein and, in solution, shows photoreversibility between the PR and PFR forms with absorption maxima at the same peaks as required for the physiological response (Fig. 4).

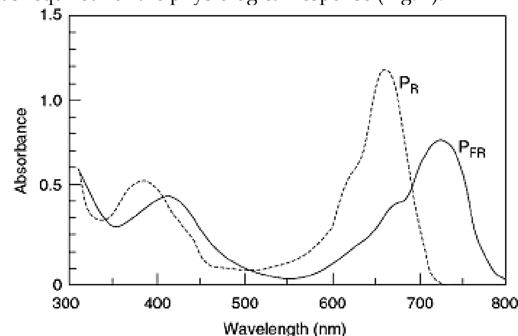


Fig: 4 The absorption spectra of phytochrome in the PR and PFR forms. PR absorbs light predominantly in the red with an absorption peak near 660 nm. PFR absorbs light at longer wavelengths, with an absorption peak near 730 nm, although significant amounts of red light are also absorbed

At its heart is a chromophore composed of a linear tetrapyrrole molecule. With specially designed equipment it is possible to detect the change in the phytochrome absorption spectrum on illumination in an intact etiolated seedling, although similar changes are masked in mature tissues which contain chlorophyll with its strong absorption peak in the red.

3.2 Low-fluence responses

The control of the germination of lettuce seeds by phytochrome was termed a low-fluence response (LFR) because it is governed by exposures to low fluences of red, or far-red, light. Many aspects of plant development exhibit low-fluence phytochrome responses including seed germination, seedling development, internode elongation and flowering in short day plants. Other key features of the LFR were determined using such experimental systems. As already described, low-fluence phytochrome responses exhibit photoreversibility. However, far-red light can reverse the effect of red light only if given within a certain period of time known as the

escape time (i.e. escape from phytochrome control). Likewise there is a delay – the lag time – following exposure to light before the response is observed. This might be a matter of a few minutes or hours, as in the case of the etiolation/de-etiolation responses, or may be days or even weeks in the case of floral induction or seed germination. Also it is the total number of photons that the sample receives, rather than the rate at which they are received, which determines whether a LFR occurs. A brief exposure to relatively intense red light has the same effect as exposure to dimmer red light for a longer period of time. This characteristic is called reciprocity, and low-fluence phytochrome responses are often reported in mmol photons m^{-2}, i.e. without a time component.

However, as the number of studies examining plant responses to phytochrome expanded it became apparent that not all phytochrome responses had the characteristics of a low-fluence response. Two other types of response, the very-low-fluence response and the high-irradiance response, were also described.

3 .3 Very-low-fluence responses

The very-low-fluence responses (VLFR) are observed only in dark-grown seedlings or imbibed seeds. They are triggered by very low fluences (hence the name!), brief exposures to light, and exhibit reciprocity.

However, they differ from the LFR in that either red or far-red light triggers the same response. For example, dormant *Arabidopsis* seeds can be triggered to germinate by red light but this is prevented by far-red light – a classic LFR. However, after 48 hours imbibition these same seeds exhibit a VLFR. They will germinate when exposed to either red or far-red light at fluences 100-1000 times lower than that required for the LFR. So how can we understand very-low-fluence responses in terms of phytochrome acting as a photointerconvertible switch? Figure 4 shows the absorption spectra of PR and PFR. Although the different forms of phytochrome have absorption maxima in thered and far-red, the peaks are quite broad and overlap. So although PR is preferentially converted to PFR by red light, a small proportion will also be converted by far-red light. In dark-grown plants phytochrome is synthesized as PR but even brief exposure to dim far-red light will convert a small amount of PR to PFR. Even though some of this will quickly be re-converted to the PR form, enough PFR is present to trigger a very-low-fluence phytochrome response. In this case phytochrome is acting as a detector of the amount of light falling on the sample without discriminating between different colours of light. Such responses are important in the very earliest responses of plants to light.

3.4 The photostationary state

The concept of phytochrome switching between different forms applies to more than just the low- and very-low-fluence responses.

Under any illumination conditions phytochrome will exist in both forms, although the precise proportion depends upon the precise spectral properties of the light. An equilibrium is established called the photostationary state. With monochromatic illumination of 660 nm and 730 nm, the proportions of PR and PFR at photoequilibrium are approximately as indicated in Table 1.

Table 1 The proportion of phytochrome (ϕ) in the P_R and P_{FR} states under different illumination conditions.

	P_R	P_{FR}	ϕ
660 nm	20%	80%	0.8
730 nm	97%	3%	0.03

The proportion of the total phytochrome which is present in the PFR form is termed f – the Greek letter Φ 'phi' is often used in biology as a shorthand for 'proportion'. Although these proportions may be constant, individual phytochrome molecules will be continually switching between the two forms. This switch is not instantaneous, and a significant proportion of the phytochrome molecules may exist in an intermediate state. There is increasing evidence that such intermediates may play an important role in phytochrome responses.

3 .5 The high-irradiance responses

Another class of phytochrome-mediated responses, the high irradiance responses (HIR), are maximized by exposure to continuous illumination, although repeated pulses of light can trigger such responses if given frequently enough. H.Mohr, working with mustard seedlings (*Sinapis alba*), found that cotyledon expansion was elicited by brief red illumination and that this was prevented by brief exposure to far-red light (a classic low-fluence response). However, if exposure to far-red light was continued for 2–3 hours the cotyledons proceeded to expand even more than under red light. This is a far-red high irradiance response (FR-HIR).

If dark-grown seedlings of most dicotyledonous plants are exposed to continuous far-red light there is a strong inhibition of seedling elongation. Typically the maximal inhibition results at far-red wavelengths (FR-HIR, Fig. 5) but this is species-dependent, with some plants exhibiting maxima in the red (R-HIR). As seedlings green these maxima often shift, indicating the operation of more than one inhibitory mechanism. Current theories favour cycling between PR and PFR as central to the operation of the HIR, but the mechanisms underlying this remain unclear. The peaks in the UV/blue of the action spectrum shown in Fig. 5 are thought to result from the action of specific UV-blue light photoreceptors which interact with phytochrome to coordinate plant development.

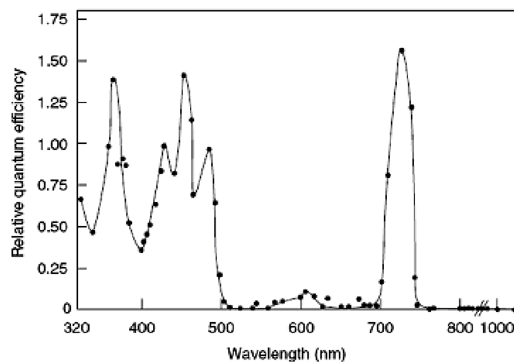


Fig: 5 Action spectrumfor the inhibition of hypocotyl growth in Lactuca sativa. Hypocotyl elongation is inhibited by far-red light in a high irradiance response (HIR). In addition, blue light is also effective: the action spectrum in the 400–500 nm region shows the typical three peaks of many blue-light responses.

3.6 What is the significance of far-red light?

Low ratios of red : far-red light are a signal of vegetation shade Full sunlight contains approximately equal amounts of all visible wavelengths of light, including red and far-red components (Fig. 1). However, plant leaves are rich in chlorophyll, which strongly absorbs blue and red light, green light to a lesser extent, but is relatively inefficient at absorbing far-red light. Fig. 6 shows the spectral composition of sunlight after it has passed through one or two leaves. Absorption of light by the canopy reduces the total irradiance markedly and has greatly reduced the amount of red light relative to far-red light. This shift in red : far-red ratio from values close to 1 in full sunlight to values nearer 0.1 is a strong signal indicating vegetation shade. This signal is not restricted to light falling directly from above. Light reflected from neighbouring vegetation will also be enriched in the far-red and has the potential to act as a signal of future competition for resources.

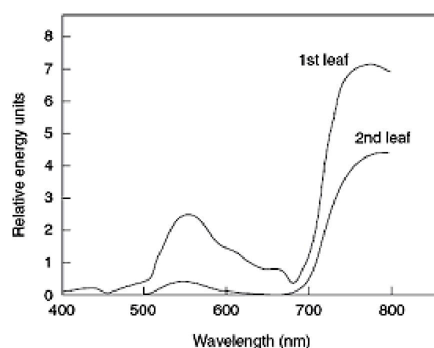


Fig: 6 The calculated spectral distribution of midday daylight transmitted through one or two leaves of sugar beet (Beta vulgaris).

Shade tolerance vs. shade avoidance

The response of plants to the red : far-red ratio is both species-specific and highly dependent upon developmental state. Consider the example of a small-seeded plant such as *Arabidopsis*. Seeds of *Arabidopsis* have limited nutrient reserves, which will support growth for only a few days. Once these reserves are exhausted the seedling must photosynthesize if it is to continue to grow. The low-fluence response of these seeds stimulates germination if at, or near, the soil surface unless the sunlight has been filtered through a vegetation canopy and is thus enriched in far-red light. If the seed does germinate, etiolation must be considered a 'last chance' response – the hypocotyls elongates rapidly until the cotyledons are above the soil. The HIR is then triggered even if the seedling is shaded. De-etiolation, resulting from exposure to far-red light, is thus a shade-tolerance response.

Some plants, e.g. dog's mercury (*Mercurialis perennis*), can tolerate shade throughout their lives and do not respond greatly to alterations in the ratio of red : far-red light. However, mature plants of many species exhibit a shade-avoidance response if exposed to elevated far-red light. Typically, the shade-avoidance response results in an increase in apical dominance, an increase in stem growth as a result of internode elongation, increased petiole elongation and accelerated flowering. The morphological changes may enable the plant to grow out of the shade of other plants. These responses can be triggered by a decrease in the ratio of red : far-red light falling from above, but can also be triggered by far-red reflected from surrounding vegetation. A single, brief exposure to far-red light can also trigger the shade-avoidance response if given at the end of the photoperiod (the 'end-of-day far-red effect', EOD-FR). Mature *Arabidopsis* plants exhibit a clear shade-avoidance response with increased petiole extension and accelerated flowering, although, as this plant grows as a rosette, the internodes do not elongate. Clearly, the shade-tolerance response of seedlings and shade-avoidance response of mature plants are antagonistic; hence a switch between the two must occur as the plant develops. The role of phytochrome in these responses is becoming increasingly clear, and these responses can be manipulated to control plant development in natural environments.

3.7 Phytochrome is encoded by a multigene family

This plethora of phytochrome responses is difficult to interpret if phytochrome is a single entity. However, it has become apparent that plants contain multiple phytochromes. Work with etiolated oat seedlings (*Avena sativa*) indicated the presence of light-labile ('Type I') and light-stable ('Type II') forms. Then Sharrock and Quail (1989) showed that *Arabidopsis* thaliana contains five phytochrome genes, which they named PHYA, PHYB, PHYC, PHYD and PHYE, and showed that whilst etiolated seedlings of *Arabidopsis* contained phyA, B and C, upon illumination there was a rapid decline in phyA. This indicated that phyAwas light-labile, corresponding to 'Type I' phytochrome described in oat, whilst phyB and C were light stable ('Type II'). PhyD and E were also demonstrated to fall into the latter class in subsequent studies.

Although the phyA content of seedlings declines rapidly upon illumination, mature plants exhibit responses attributable to phyA action; therefore a small amount

must still remain in light-grown plants. The expression of phyA is regulated by light by both transcriptional and post-transcriptional processes. The transcription of the PHYA gene decreases markedly (but is not abolished) upon illumination. At the post-transcriptional level phyA protein is readily degraded in the light after its photoconversion to the phyAFR form. Most plants contain multiple phytochromes, which appear to have arisen by gene duplication. For example tomato (*Lycopersicon esculentum*) contains five (or perhaps six) phytochrome genes. The nomenclature has become somewhat confused, but comparisons of phytochrome amino acid sequences have indicated that four major subfamilies, each with one or more members, can be identified in herbaceous dicots. These subfamilies have been named PHYA, PHYB, PHYC and PHYE, based on comparisons with the genes from *Arabidopsis*. PHYD is closely related to PHYB and is placed in the same subfamily. In some species, e.g. black cottonwood (*Populus trichocarpa*), not all of the subfamilies have been detected. Monocots also contain multiple phytochromes, although an extensive survey of grasses by Mathews and Sharrock (1996) found evidence of only the PHYA, B and C subfamilies.

Unravelling the roles of these different phytochromes is a challenging task but has been greatly facilitated by the use of mutant plants which have lesions in their phytochrome genes. Likewise the use of transgenic plants where the content of an individual phytochrome has been increased has been highly informative.

Screening for *Arabidopsis* plants which exhibited elongated hypocotyls when grown under white light revealed a number of different mutants, the hy mutants. The mutants hy1, hy contain phytochrome apoprotein but do not contain the chromophore – these are mutations affecting tetrapyrrole synthesis. Therefore these mutants are often considered to lack all functional phytochrome, although some residual activity may remain. The hy$_3$ mutant lacks PHYB and the hy5 mutant has a lesion in the phytochrome signal transduction chain rather than in a phytochrome gene itself. Although many of these initial mutations exhibited lesions in aspects of phytochrome responses, because the screen was performed under white light other photomorphogenic mutants were also identified. The power of this approach was amply demonstrated when it was found that the hy$_4$ mutant contained a lesion in the blue-light receptor which had previously proved impossible to identify. More directed screens using continuous far-red illumination identified plants with lesions in the PHYA gene, and other molecular genetic approaches have been used to identify *Arabidopsis* plants with lesions in PHYC, D and E. Such mutants allow the functions of individual phytochromes to be assessed, and many plants with lesions in the phytochrome signal transduction chain have also been isolated.

Our understanding of the phytochrome gene family is currently most advanced in *Arabidopsis* but mutations have also been found in other species which are often more useful in ecophysiological studies including pea (*Pisum spp.*), tomato, cucumber (*Cucumis sativus*), tobacco (*Nicotiana tabacum*), *Brassica napus* and *Sorghum bicolor*.

4 UV-A/blue light photoreceptors (cryptochrome)

Traditional biochemical approaches were used firstly to isolate the phytochrome protein. This strategy succeeded because the unique spectral properties of phytochrome enabled it to be identified in subcellular fractions even though it is a very low abundance protein. This approach failed with the UV-A/blue light photoreceptor as many other proteins within the cell, which have no role in photoperception, absorb blue light owing to bound cofactors. However, the identification of a mutant (hy$_4$) which had a lesion in blue-light responses allowed the gene encoding a UV-A/blue photoreceptor to be identified. This mutant was renamed cry1 as cryptochrome (crypto = hidden) is an alternative name for this class of photoreceptors.

The *Arabidopsis* CRY1 gene has been sequenced and encodes a protein with similarity to a class of microbial DNA repair enzymes called DNA photolyases. Whilst biochemical measurements of the plant protein showed no evidence of DNA repair activity, these enzymes are known to bind a flavin cofactor (flavin adenine dinucleotide) which absorbs blue light, consistent with its proposed role as a blue-light receptor. It has also been suggested that cry1 binds a second chromophore – a pterin (5,10 methenyltetrahydrofolate). The identification of CRY1 in *Arabidopsis* quickly led to the identification of a second, related gene called CRY2 , indicating that the cryptochromes are encoded by a small, multigene family. Likewise, two related cryptochrome genes have recently been identified in tomato.

Plants contain other, unrelated, blue-light receptors in addition to the cryptochromes. Blue-light phototropic curvatures, chloroplast and stomatal movements are stimulated, in part, by phototropin(s) and a separate, as yet unidentified, photoreceptor most probably mediates responses to UV-B.

5 Genes controlling etiolated growth

Clearly, the switch from etiolated to de-etiolated growth requires many aspects of plant development to change. The emphasis in this chapter so far has been on the development of plants in the light (photomorphogenesis) but it is equally important to remember that seedlings are following a developmental pathway when growing in darkness. This is sometimes referred to as skotomorphogenesis (skoto = dark). Just as plants with mutations in light perception have proved invaluable in understanding photomorphogenesis, valuable insights into skotomorphogenesis have been obtained by isolating plants which exhibit some, or all, of the characteristics of de-etiolated plants even though they have not been exposed to light. A number of laboratories have isolated such mutants, calling them det (de-etiolated) and cop (constitutively photomorphogenic). A third class of mutants, fus (fusca), also develop in a de-etiolated manner when grown in darkness but were originally isolated on the basis of increased anthocyanin accumulation when grown in the light. Many of these mutants (involving lesions in at least 11 different genes) show an almost complete photomorphogenic development in total darkness. They have short hypocotyls, the cotyl edons open and true leaves form, chloroplasts develop and many light-regulated genes are

expressed. It is quite remarkable that these plants can complete their entire life cycle in darkness, from germination to flowering and seed production, if provided with a suitable nutrient source (e.g. glucose). They do not green under these conditions, however, as the biosynthesis of chlorophyll includes a light-dependent step. The det, cop and fus genes are obviously fundamental in maintaining the seedling in an etiolated state. Other mutations result in only partial photomorphogenesis in darkness.

Some of the genes which have been mutated in these plants have been identified and sequenced, providing valuable insights into this aspect of plant development. The det_2 mutant contains a lesion in the gene encoding a cytochrome P450. This class of enzymes plays a central role in many aspects of secondary metabolism and plants contain many different cytochrome P 450s (at least 30 in *Arabidopsis*).

This particular cytochrome P450 is required for brassinosteroid biosynthesis and the phenotype of this mutant reverts to that of the wild type if supplied with appropriate brassinosteroid precursors. *Det2* mutants grown in the light are severely dwarfed, indicating that these compounds are important in both dark- and light-regulated developmental pathways. A number of other dwarf plants have since been found to have lesions in other parts of the brassinosteroid biosynthetic pathway. Interestingly, wild-type plants treated with cytokinins also develop in a de-etiolated manner when grown in darkness, although the significance of this is not yet clear.

Other det/cop/fus mutations are thought to be components of a signal transduction chain involved in both skoto- and photomorphogenesis.

6 Unravelling photomorphogenesis

Given that plants contain so many light-responsive pathways, the ability to inactivate individual components of specific pathways selectively provides a powerful tool with which to study these aspects of plant biology. This approach can be readily extended to examine multiple pathways by crossing different mutants together and selecting offspring which have lesions in two, three or even more photoreceptors. This is amply demonstrated in the examples described in the following section.

6.1 Phytochrome A and B regulate seed germination in *Arabidopsis*

As described earlier, the germination of recently imbibed seeds of *Arabidopsis* is controlled by a low-fluence phytochrome response (LFR) whilst imbibition for $_4$ ^{0} hours results in a shift to control by a very-low-fluence response (VLFR). In phy B-deficient mutants the initial photoreversible LFR is abolished. This tells us not only that phyB is responsible for the LFR but also that the other phytochromes (phyA, phyC–E) cannot substitute for it. With phyA deficient mutants the situation is reversed. The VLFR is lost, showing that this response requires phyA. Since wenowhave seeds which show just one or other response we can measure the properties of the lowand very-low-fluence responses independently (Fig. 7). The VLFR is triggered by 1–1000nmolm^{-2} (at 660nm) and is not photoreversible. The action spectrum closely resembles the absorption spectrum of phyAR, which is consistent with the view that absorption of light by phyAR leads to the formation of phyAFR, which triggers the response. In contrast, the LFR requires much more light (10–1000 mmol m^{-2}), is stimulated by red light (550–690nm), and reversed by far-red light (700–800 nm).

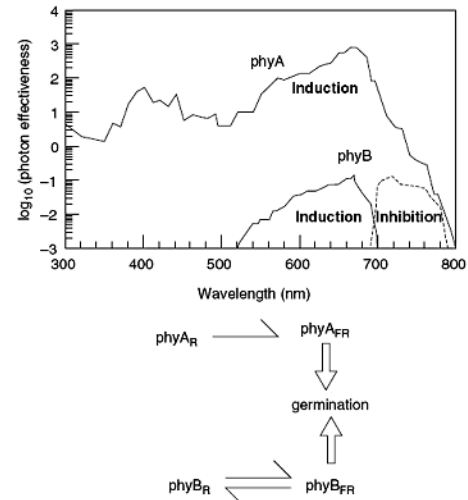


Fig: 7 Phytochrome A and B regulate seed germination in Arabidopsis. In the absence of phyB, the action spectrum for phyAdependent germination is revealed. All wavelengths of light induce germination, although the response is optimal in the red (note the log scale). This is a characteristic verylow- fluence response. In the absence of phyA, the action spectrum for phyB-dependent germination is apparent. This is a low-fluence response: red light stimulates germination whilst farred light is inhibitory. The different responses to phyA and phyB can be separated only in the mutant plants.

In this example the different responses can be attributed quite readily to the action of either phyA or phyB, and there appears to be little interaction with either each other or cryptochrome. However, in many cases, interactions within and between the families of photoreceptors is the norm. The responses of seedlings to light are complex, and our understanding of the regulation of de-etiolation is far from complete.

6.2 Cryptochrome and phytochrome interact to regulate de-etiolation

When *Arabidopsis* seedlings are grown under continuous far-red light, a high-irradiance response causes them to de-etiolate. However, phyA-deficient plants are etiolated in all respects under these conditions, clearly identifying phyA as an essential component of the HIR signal transduction pathway.

For seedlings grown under continuous red light the situation is more complex. Someaspects of red-light-induced de-etiolation, such as the inhibition of hypocotyl elongation, can be attributed to phyB. However, other responses, such as the opening of the apical hook, can be mediated by phyA or phyB. Experiments under a variety of illumination conditions have shown that apical hook

opening (and other de-etiolation responses) are controlled bymultiple redundant pathways (Table 2).

In an attempt to unravel the roles of different photoreceptors in the control of de-etiolation responses Neff & Chory (1998) examined the development of *Arabidopsis* seedlings with lesions in phyA, phyB and cry1. The single mutants were also crossed together so that additional plants with double and triple mutations could be studied. Whereas the studies of seed germination measured an 'all-or-nothing' response (i.e. a seed germinated or it did not), de-etiolation is much more complex, consisting ofmultiple responses, each ofwhichmay be quantified precisely.

Table 2 Redundant interactions between phyA, phyB and cry1. Apical hook opening can be achieved under various light regimes, mediated by different photoreceptors and pathways.

Illumination	Photoreceptors
Far-red	phyA, (phyB[a])
Red	phyA, phyB
Blue	phyA, phyB, cry1

[a]partial response only

Wild-type plants which have been grown in white light for five days have short hypocotyls, an open apical hook and open, expanded cotyledons which have accumulated chlorophyll and anthocyanin. In contrast, dark-grown plants have a much longer hypocotyl, a welldeveloped apical hook and folded, unexpanded cotyledons without chlorophyll and anthocyanin (Table 3).

The triple mutant lacks cry1, phyA and phyB. When grown in white light some de-etiolation responses are entirely abolished. For example, the apical hook, cotyledon expansion and anthocyanin content are the same as those of a wild-type plant grown in darkness, which suggests that these three photoreceptors control the development of these features. However, the cotyledons have opened slightly and a small amount of chlorophyll is still produced, indicating that other photoreceptors have a minor role in de-etiolation (other studies have implicated phyD). The hypocotyl is even longer than that of an etiolated wild-type plant, probably because limited photosynthesis can occur, providing additional energy for growth. This approach allows the potential role of individual photoreceptors to be investigated, although care must be taken in interpreting the results. When one photoreceptor has been inactivated by mutagenesis, another may take over its function. However, just because a photoreceptor can substitute for another is not necessarily proof that it does fulfil the same role in a normal plant. Other studies have shown that the interaction between photoreceptors may be complex. For example, the action of one photoreceptor may be greatly enhanced by the activity of another, beyond that expected from simple additive effects.

Table 3 Phenotypes of wild-type and photoreceptor mutants of *Arabidopsis* when grown in the light or dark. In many ways (but not all), plants lacking phytochromes A, B and cryptochrome 1 grown in the light resemble dark-grown plants.

Genotype	Angle (degrees)		Cotyledon area (mm^2)	Hypocotyl length (mm)	Chlorophyll content (μg seedling^{-1})	Anthocyanin content (Units seedling^{-1})
	Hook	Cotyledon				
Wild type (light)	172	206	2.2	3.1	0.76	0.75
Wild type (dark)	132	15	0.2	9.7	not detected	not detected
phyAphyBcry1 (light)	132	38	0.2	13.9	0.19	not detected

6.3 Phytochrome responses throughout the life cyle

Wild-type seedlings of *Arabidopsis* exhibit shade tolerance (i.e. they initially de-etiolate when exposed to far-red light) whilst mature plants exhibit shade avoidance (the leaf petioles elongate and flowering is accelerated under far-red light). Therefore the response of the plant clearly depends upon its developmental state. The photoreceptor mutants have helped us to understand how these different responses are controlled at different stages of the plants' life cycle. When a seedling first develops it contains relatively high amounts of phyA and phyB. Therefore the seedling will de-etiolate under red light (a LFRmediated by phyB) and far-red light (a HIR mediated by phyA) and is thus shade tolerant. However, continued illumination leads to a decline in phyA content. The seedling will remain de-etiolated under red light (due to the continued action of phyB) but will now etiolate under far-red light; i.e. it now exhibits shade avoidance.

A phyB-deficient plant will exhibit a constitutive shade-avoidance response both as a seedling and a mature plant. Under white light the phyB mutant has elongated petioles but if exposed to a 10-minute pulse of far-red light at the end of the day, a more extreme shade-avoidance response is triggered, indicating that other photoreceptors are still responsive. If phyD is also mutated, the petioles become further elongated under white light, whilst mutations in phyE cause the rosette habit to be lost. The role of these minor phytochromes is normally masked by phyB. Although mature plants of many species do not exhibit shade tolerance, it can be re-established using genetic engineering techniques. Although the phyA content of a mature plant is normally low, an artificial gene can be made where a strong, unregulated promoter is fused to the coding region of the PHYA gene. When this over-expression construct is introduced into plants they will nowcontain much more phyA than normal. Vierstra and coworkers used this approach to produce shade-tolerant tobacco plants. Wild-type tobacco plants normally show a strong shade-avoidance response when they are illuminated with far-red light from the side, or are grown in dense canopies. The internodes elongate and the specific leaf area (the area of leaf per unit dry weight) increases; i.e. the leaves become thinner. These responses were abolished in transgenic plants which contained five times asmuch phyA as the wild type. However, increasing the phyA content by this much resulted in dwarf plants, presumably because they became hypersensitive to far-red light. Smith and coworkers found that transgenic tobacco plants which contained lower amounts of oat phyA developed normally under white light, but showed a reduced shade avoidance response. This ability to manipulate plant responses subtly to shade has obvious agricultural applications as it allows plants to be grown closer together.

7 Phytochrome signal transduction

So far our discussion of photomorphogenesis has focused on signal perception (i.e. the photoreceptors) and the responses observed. Clearly, a complex set of signal

transduction pathways must link the two. Although our understanding of these pathways is far from complete, we can trace certain components of them. Our understanding of phytochrome signal transduction is considerably more advanced than that of cryptochrome, so this will be discussed further.

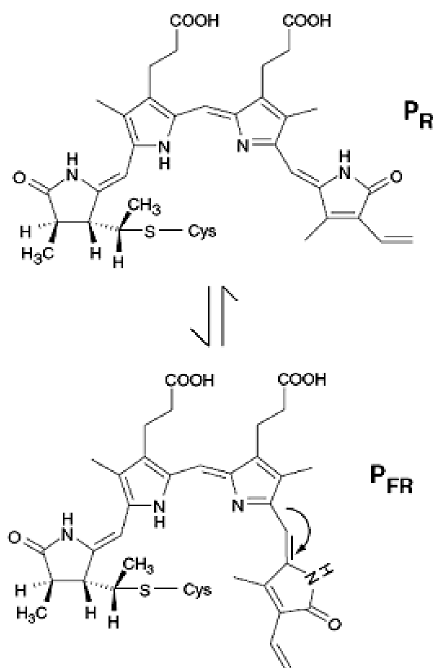


Fig: 8 The phytochrome chromophore undergoes a cis–trans isomerization as phytochrome is converted from the PR to PFR form.The phytochrome chromophore is a linear tetrapyrrole molecule covalently linked to the phytochrome apoprotein via a cysteine residue (Cys). One of the bonds linking the pyrrole rings moves as phytochrome is illuminated (arrow).

7.1 How does phytochrome detect light?

The first step of photoperception by phytochrome is the absorption of a photon by the chromophore, but how does this initiate subsequent responses? The chromophore is a linear tetrapyrrole molecule, covalently attached to the phytochrome apoprotein, and it undergoes a reversible conformational change when it absorbs light. In PR, each bond linking the pyrrole groups is in the cis configuration (Fig. 8). Upon conversion to PFR, one of these bonds shifts to the trans configuration, i.e. part of the chromophore has moved. We do not know precisely how this movement initiates subsequent events, but it alters the absorption spectrum, and presumably other properties, of the protein. We do know that both phyA and phyB interact with other proteins, and that both possess a kinase activity – they can phosphorylate themselves (autophosphorylation) and other proteins. A number of proteins have been identified which interact with phytochrome, including PIF_3 (phytochrome interacting factor 3). The gene encoding PIF_3 was also found, in an independent set of experiments, to be mutated in plants which exhibited impaired responses to red and far-red light. PIF_3 is, therefore, likely to be an important component of the phytochrome signal transduction chain. Mutagenic studies have identified many plants with lesions in phytochrome signal transduction chains, indicating that many proteins play a role in signal transduction. In some cases mutations affect responses to just phytochrome A or B, whereas other mutants affect responses to both phytochromes. In addition, microinjection and pharmacological studies have implicated a number of small molecules and other proteins in early signalling processes including Ca^{2+}, cyclic AMP and calmodulin (a protein which binds Ca^{2+}). Again, the role of these compounds is not yet understood but, by analogy with other systems, they are likely to have a role in very rapid responses occurring immediately following phytochrome activation.

7.2 Phytochrome A and B move into the nucleus upon illumination

Many of the changes in plant development which occur during photomorphogenesis result from changes in gene expression. Many hundreds, if not thousands, of genes are light-regulated, which is not surprising given that light triggers the development of the photosynthetic apparatus and the expression of many enzymes of metabolism. The majority of these light-regulated genes are located in the nucleus, although a proportion of the photosynthetic apparatus is encoded in the chloroplast genome. The regulation of chloroplast gene expression is quite different from that of nuclear-encoded genes.

Early studies proposed that a proportion of phytochrome was associated with membranes, but this is now thought either to be an artefact resulting from the preparation of cell extracts or to represent a pool of physiologically irrelevant phytochrome in the process of being degraded. Current theories favour a model where phytochrome A and B are initially located in the cytoplasm and then move into the nucleus upon activation. Studies of this movement are possible because phytochrome is still physiologically active even if fused to another protein. Genetically modified plants have been produced where the coding region of PHYA or PHYB has been fused to a reporter gene. One such reporter gene encodes green fluorescent protein (GFP) which fluoresces green when illuminated with blue light, allowing its position to be determined by microscopy. When plants are grown in darkness, phyA::GFP fusions (or phyB::GFP fusions) are found in the cytoplasm. When treated with light which will trigger a phytochrome response, the fusion proteins move into the nucleus. The movement of phyB::GFP is rapid (it occurs within 15 minutes), red : far-red reversible and requires the presence of the chromophore. The phyA::GFP fusion protein enters the nucleus under illumination conditions which would trigger VLFR and HIR. Having entered the nucleus the phytochromes accumulate in small patches or 'speckles'. In contrast, phy C, D and E are constitutively localized in the nucleus, although speckle formation results fromillumination with red light and can be reversed by far-red light.

At least two pathways have been identified by which phytochrome can stimulate nuclear gene expression, and others undoubtedly exist. In both cases, phytochrome

enters the nucleus and interacts with other proteins, and these then stimulate gene expression by binding to short stretches of DNA (light-responsive-elements – LREs) found in the promoters of light-responsive genes. In this way the expression of many different genes can be stimulated by the action of a few signal transduction chains.

The first pathway involves a number of proteins whose role was first identified in mutagenic studies (Fig. 9). These include COP1, other members of the cop/det/fus family, and HY5. In the dark, a large multiprotein complex (referred to as a signalosome) is found in the nucleus which includes the COP1 protein. This interacts with HY5 and prevents HY5 from binding to the LREs in the promoters of lightregulated genes. In the absence of HY5, transcription does not occur. When phytochrome is activated, it enters the nucleus and breaks down the association between HY5, COP1 and the signalosome. HY5 binds to the LREs and the transcription of light-regulated genes is stimulated, triggering de-etiolation. COP1 leaves the nucleus and enters the cytoplasm, although the rest of the signalosome remains.

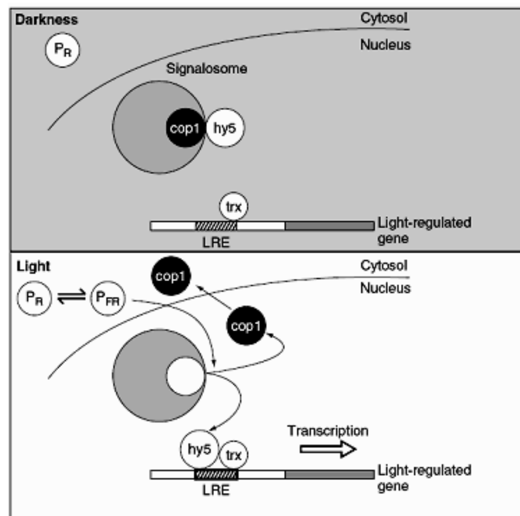


Fig: 9 Signalling and photomorphogenesis. A number of signalling pathways have been identified which result in lightactivated gene expression, one of which is shown here. In the dark, phytochrome is in the PR form and located in the cytosol. In the nucleus a large multiprotein complex, known as a signalosome, binds HY5. Light-regulated genes are not active. Upon illumination, some phytochrome is converted to the PFR form. This enters the nucleus and disrupts the interaction between a component of the signalosome, COP1, and HY5. HY5 is now free to bind to DNA sequences known as light responsive elements (LREs) found in the promoters of light-regulated genes, triggering transcription.

This model elegantly explains the phenotypes of the various mutants. Plants which lack phytochrome or HY5 will be etiolated in the light as the transcription of the light-regulated genes is not stimulated. On the other hand, the absence of COP1, or other components of the signalosome, causes the plants to be constitutively deetiolated as HY5 is always able to stimulate transcription. The second pathway involves PIF_3. As well as interacting with phytochrome, PIF_3 will also bind to another class of LRE. Although PIF_3 will bind to the LRE in both the light and the dark, it forms

a complex with phytochrome only in the PFR form. This complex of PIF_3 and PFR is thought to regulate the recruitment of other protein factors required for transcription.

These models go some way to illustrate how phytochrome can trigger gene expression, but undoubtedly much remains to be discovered. Many other proteins can interact with these signal transduction chains and some light-regulated genes are regulated by other pathways. However, the principles of these models are likely to be repeated in other signal transduction chains.

7.3 Photoreceptors' interactions with plant growth hormones

Although the emphasis of this chapter has been on the perception of light by photoreceptors and the ways in which these signals affect gene expression, and subsequently plant development, photomorphogenesis represents a profound change in plant development. It is not surprising therefore that plant growth hormones play a central role in mediating many of these responses. We have already seen how cytokinin and brassinosteroids influence skotomorphogenesis. Recently, a protein induced by cytokinin treatment (ARR4) has been shown to bind to PhyB and stabilize the FR form and over-expression of ARR4 results in plants hypersensitive to red light. This demonstrates a direct link between a photoreceptor and a plant growth hormone.

Another example is provided by the interaction between the shade-avoidance response and ethylene. Ethylene production in sorghum (*Sorghum bicolor*) is under circadian control, the peak amplitude of which is strongly increased by shade conditions. A phyB mutant of sorghum that exhibits a constitutive shade-avoidance response also produces much more ethylene than normal, even when grown under full sunlight. In tobacco, Pierik et al. (2003) have demonstrated that many features of the shade-avoidance response can be stimulated by exposing plants to ethylene, and that ethylene-insensitive tobacco plants show a delayed shade-avoidance response when grown at high densities or when exposed to EOD-FR light. The molecular basis of this interaction is yet to be understood.

As auxin and gibberellic acid are important in stimulating plant growth, it is to be expected that there will be many interactions between these plant growth hormones and light.

As our understanding of the signal transduction chains of individual photoreceptors improves, the interactions between them will become clearer. The challenge will still remain of integrating this detailed molecular information into a broader ecophysiological context.

8. Ecological functions: Circadian rhythms

Various metabolic processes in plants, such as oxygen evolution and respiration, cycle alternately through high-activity and low-activity phases with a regular periodicity of about 24 hours. These rhythmic changes are referred to as **circadian rhythms** (from the Latin *circa diem*, meaning "approximately a day"). The **period** of a rhythm is the time that elapses between successive peaks or troughs in the cycle, and because the rhythm persists in the absence of external controlling factors, it is considered to be **endogenous**.

The endogenous nature of circadian rhythms suggests that they are governed by an internal pacemaker, called an **oscillator**. The endogenous oscillator is coupled to a variety of physiological processes. An important feature of the oscillator is that it is unaffected by temperature, which enables the clock to function normally under a wide variety of seasonal and climatic conditions. The clock is said to exhibit **temperature compensation**.

Light is a strong modulator of rhythms in both plants and animals. Although circadian rhythms that persist under controlled laboratory conditions usually have periods one or more hours longer or shorter than 24 hours, in nature their periods tend to be uniformly closer to 24 hours because of the synchronizing effects of light at daybreak, referred to as **entrainment**. Both red and blue light are effective in entrainment. The red-light effect is photoreversible by far-red light, indicative of phytochrome; the blue-light effect is mediated by blue-light photoreceptor(s).

8.1 Phytochrome Regulates the Sleep Movements of Leaves

The sleep movements of leaves, referred to as **nyctinasty**, are a well-described example of a plant circadian rhythm that is regulated by light. In nyctinasty, leaves and/or leaflets extend horizontally (open) to face the light during the day and fold together vertically (close) at night (Figure 17.12). Nyctinastic leaf movements are exhibited by many legumes, such as *Mimosa*, *Albizia*, and *Samanea*, as well as members of the oxalis family. The change in leaf or leaflet angle is caused by rhythmic turgor changes in the cells of the **pulvinus** (plural *pulvini*), a specialized structure at the base of the petiole.

Once initiated, the rhythm of opening and closing persists even in constant darkness, both in whole plants and in isolated leaflets (Figure 10). The phase of the rhythm, however, can be shifted by various exogenous signals, including red or blue light. Light also directly affects movement: Blue light stimulates closed leaflets to open, and red light followed by darkness causes open leaflets to close. The leaflets begin to close within 5 minutes after being transferred to darkness, and closure is complete in 30 minutes. Because the effect of red light can be canceled by far-red light, phytochrome regulates leaflet closure.

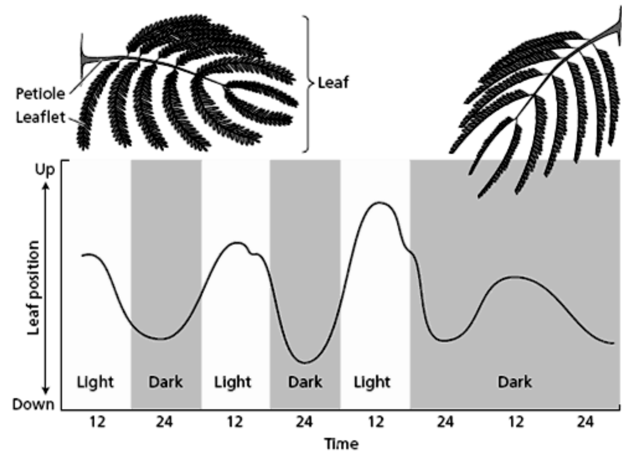


FIGURE 10 Circadian rhythm in the diurnal movements of *Albizia* leaves. The leaves are elevated in the morning and lowered in the evening. In parallel with the raising and lowering of the leaves, the leaflets open and close. The rhythm persists at a lower amplitude for a limited time in total darkness.

The physiological mechanism of leaf movement is well understood. It results from turgor changes in cells located on opposite sides of the pulvinus, called **ventral motor cells** and **dorsal motor cells** (Figure 11). These changes in turgor pressure depend on K^+ and Cl^- fluxes across the plasma membranes of the dorsal and ventral motor cells. Leaflets open when the dorsal motor cells accumulate K^+ and Cl^-, causing them to swell, while the ventral motor cells release K^+ and Cl^-, causing them to shrink. Reversal of this process results in leaflet closure. Leaflet closure is therefore an example of a rapid response to phytochrome involving ion fluxes across membranes.

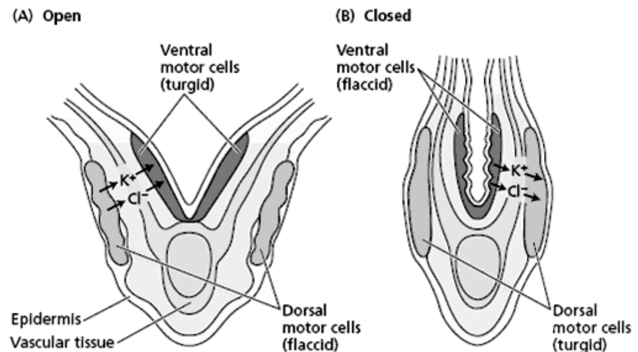


FIGURE 11 Ion fluxes between the dorsal and ventral motor cells of *Albizia pulvini* regulate leaflet opening and closing.

Gene expression and circadian rhythms.

Phytochrome can also interact with circadian rhythms at the level of gene expression. The expression of genes in the *LHCB* family, encoding the lightharvesting chlorophyll *a/b*–binding proteins of photosystem II, is regulated at the transcriptional level by both circadian rhythms and phytochrome. In leaves of pea and wheat, the level of *LHCB* mRNAhas been found to oscillate during daily light–dark cycles, rising in the morning and falling in the evening. Since the rhythm persists even in continuous darkness, it appears to be a circadian rhythm. But

phytochrome can perturb this cyclical pattern of expression.

When wheat plants are transferred from a cycle of 12 hours light and 12 hours dark to continuous darkness, the rhythm persists for a while, but it slowly *damps out* (i.e., reduces in amplitude until no peaks or troughs are discernible). If, however, the plants are given a pulse of red light before they are transferred to continuous darkness, no damping occurs (i.e., the levels of *LHCB* mRNA continue to oscillate as they do during the light–dark cycles). In contrast, a far-red flash at the end of the day prevents the expression of *LHCB* in continuous darkness, and the effect of far red is reversed by red light. Note that it is not the oscillator that damps out under constant conditions, but the coupling of the oscillator to the physiological event being monitored. Red light restores the coupling between the oscillator and the physiological process.

8.2 Circadian Clock Genes of *Arabidopsis* Have Been Identified

The isolation of clock mutants has been an important tool for the identification of clock genes in other organisms. Isolating clock mutants in plants requires a convenient assay that allows monitoring of the circadian rhythms of many thousands of individual plants to detect the rare abnormal phenotype.

To allow screening for clock mutants in *Arabidopsis*, the promoter region of the *LHCB* gene was fused to the gene that encodes luciferase, an enzyme that emits light in the presence of its substrate, luciferin. This reporter gene construct was then used to transform *Arabidopsis* with the Ti plasmid of *Agrobacterium* as a vector. Investigators were then able to monitor the temporal and spatial regulation of bioluminescence in individual seedlings in real time using a video camera.

A total of 21 independent ***toc*** (*t*iming *o*f *C*AB [*LHCB*] expression) mutants have been isolated, including both short-period and long-period lines. The *toc1* mutant in particular has been implicated in the core oscillator mechanism.

9. Blue Light Stimulates Stomatal Opening

We now turn our attention to the stomatal response to blue light. Stomata have a major regulatory role in gas exchange in leaves, and they can often affect yields of agricultural crops. Several characteristics of blue light–dependent stomatal movements make guard cells a valuable experimental system for the study of bluelight responses:

- The stomatal response to blue light is rapid and reversible, and it is localized in a single cell type, the guard cell.
- The stomatal response to blue light regulates stomatal movements throughout the life of the plant. This is unlike phototropism or hypocotyl elongation, which are functionally important at early stages of development.
- The signal transduction cascade that links the perception of blue light with the opening of stomata is understood in considerable detail.

In the following sections we will discuss two central aspects of the stomatal response to light, the osmoregulatory mechanisms that drive stomatal movements, and the role of a blue light–activated H^+-ATPase in ion uptake by guard cells. Light is the dominant environmental signal controlling stomatal movements in leaves of well-watered plants growing in natural environments. Stomata open as light levels reaching the leaf surface increase, and close as they decrease. In greenhouse-grown leaves of broad bean (*Vicia faba*), stomatal movements closely track incident solar radiation at the leaf surface.

Early studies of the stomatal response to light showed that DCMU (dichlorophenyldimethylurea), an inhibitor of photosynthetic electron transport (see Figure 7.31), causes a partial inhibition of light-stimulated stomatal opening. These results indicated that photosynthesis in the guard cell chloroplast plays a role in light-dependent stomatal opening, but the observation that the inhibition was only partial pointed to a nonphotosynthetic component of the stomatal response to light. Detailed studies of the light response of stomata have shown that light activates two distinct responses of guard cells: photosynthesis in the guard cell chloroplast, and a specific blue-light response.

The specific stomatal response to blue light cannot be resolved properly under blue-light illumination because blue light simultaneously stimulates both the specific blu-light response and guard cell photosynthesis (for the photosynthetic response to blue light, see the action spectrum for photosynthesis in Figure 7.8). Aclear-cut separation of the responses of the two light responses can be obtained in dual-beam experiments. High fluence rates of red light are used to *saturate* the photosynthetic response, and low photon fluxes of blue light are added after the response to the saturating red light has been completed. The addition of blue light causes substantial further stomatal opening that cannot be explained as a further stimulation of guard cell photosynthesis because photosynthesis is saturated by the background red light.

An action spectrum for the stomatal response to blue light under background red illumination shows the three finger pattern discussed earlier. This action spectrum, typical of blue-light responses and distinctly different from the action spectrum for photosynthesis, further indicates that, in addition to photosynthesis, guard cells respond specifically to blue light.

When guard cells are treated with cellulolytic enzymes that digest the cell walls, *guard cell protoplasts* are released. Guard cell protoplasts *swell* when illuminated with blue light, indicating that blue light is sensed within the guard cells proper. The swelling of guard cell protoplasts also illustrates how intact guard cells function. The light-stimulated uptake of ions and the accumulation of organic solutes decrease the cell's osmotic potential (increase the osmotic pressure). Water flows in as a result, leading to an increase in turgor that in guard cells with intact walls is mechanically transduced into an increase in stomatal apertures. In the absence of a cell wall, the blue light–mediated increase in osmotic pressure causes the guard cell protoplast to swell.

9.1 Blue Light Activates a Proton Pump at the Guard Cell Plasma Membrane

When guard cell protoplasts from broad bean (*Vicia faba*) are irradiated with blue light under background red-light illumination, the pH of the suspension medium becomes more acidic (Figure 18.13). This blue light–induced acidification is blocked by inhibitors that dissipate pH gradients, such as CCCP, and by inhibitors of the proton-pumping H^+-ATPase, such as vanadate This indicates that *the acidification results from the activation by blue light of a proton-pumping ATPase in the guard cell plasma membrane* that extrudes protons into the protoplast suspension medium and lowers its pH. In the intact leaf, this blue-light stimulation of proton pumping lowers the pH of the apoplastic space surrounding the guard cells. The plasma membrane ATPase from guard cells has been isolated and extensively characterized.

9.2 Blue Light Regulates Osmotic Relations of Guard Cells

Blue light modulates guard cell osmoregulation via its activation of proton pumping (described earlier) and via the stimulation of the synthesis of organic solutes. Before discussing these blue-light responses, let us briefly describe the major osmotically active solutes in guard cells. The botanist Hugo von Mohl proposed in 1856 that turgor changes in guard cells provide the mechanical force for changes in stomatal apertures. The plant physiologist F. E. Lloyd hypothesized in 1908 that guard cell turgor is regulated by osmotic changes resulting from starch–sugar interconversions, a concept that led to a starch–sugar hypothesis of stomatal movements. The discovery of the changes in potassium concentrations in guard cells in the 1960s led to the modern theory of guard cell osmoregulation by potassium and its counterions.

Potassium concentration in guard cells increases severalfold when stomata open, from 100 m*M*in the closed state to 400 to 800 m*M*in the open state, depending on the plant species and the experimental conditions. These large concentration changes in the positively charged potassium ions are electrically balanced by the anions Cl^- and malate^{2-} (Figure 12A). In species of the genus *Allium*, such as onion (*Allium cepa*), K^+ ions are balanced solely by Cl^-. In most species, however, potassium fluxes are balanced by varying amounts of Cl^- and the organic anion malate^{2-} .

The Cl^- ion is taken up into the guard cells during stomatal opening and extruded during stomatal closing. Malate, on the other hand, is synthesized in the guard cell cytosol, in a metabolic pathway that uses carbon skeletons generated by starch hydrolysis (see Figure 12B). The malate content of guard cells decreases during stomatal closing, but it remains to be established whether malate is catabolized in mitochondrial respiration or is extruded into the apoplast. Potassium and chloride are taken up into guard cells via secondary transport mechanisms driven by the gradient of electrochemical potential for H^+, $\Delta m H^+$, generated by the proton pump discussed earlier in the chapter. Proton extrusion makes the electric-potential difference across the guard cell plasma membrane more negative; light-dependent hyperpolarizations as high as 50 mV have been measured. In addition, proton pumping generates a pH gradient of about 0.5 to 1 pH unit.

The electrical component of the proton gradient provides a driving force for the passive uptake of potassium ions via voltage-regulated potassium channels. Chloride is thought to be taken up through anion channels. Thus, blue light–dependent stimulation of proton pumping plays a key role in guard cell osmoregulation during light-dependent stomatal movements

Guard cell chloroplasts contain large starch grains, and their starch content decreases during stomatal opening and increases during closing. Starch, an insoluble, high-molecular-weight polymer of glucose, does not contribute to the cell's osmotic potential, but the hydrolysis of starch into soluble sugars causes a decrease in the osmotic potential (or increase in osmotic pressure) of guard cells. In the reverse process, starch synthesis decreases the sugar concentration, resulting in an increase of the cell's osmotic potential, which the starch–sugar hypothesis predicted to be associated with stomatal closing. With the discovery of the major role of potassium and its counterion in guard cell osmoregulation, the sugar– starch hypothesis was no longer considered important. Recent studies, however, described in the next section, have characterized a major osmoregulatory phase of guard cells in which sucrose is the dominant osmotically active solute.

Sucrose Is an Osmotically Active Solute in Guard Cells

Studies of daily courses of stomatal movements in intact leaves have shown that the potassium content in guard cells increases in parallel with early-morning opening, but it decreases in the early afternoon under conditions in which apertures continue to increase. The sucrose content of guard cells increases slowly in the morning, but upon potassium efflux, sucrose becomes the dominant osmotically active solute, and stomatal closing at the end of the day parallels a decrease in the sucrose content of guard cells (Figure 13).

These osmoregulatory features indicate that stomatal opening is associated primarily with K^+ uptake, and closing is associated with a decrease in sucrose content. The need for distinct potassium- and sucrose dominated osmoregulatory phases is unclear, but it might underlie regulatory aspects of stomatal function. Potassium might be the solute of choice for the consistent daily opening that occurs at sunrise. The sucrose phase might be associated with the coordination of stomatal movements in the epidermis with rates of photosynthesis in the mesophyll. Where do osmotically active solutes originate? Four distinct metabolic pathways that can supply osmotically active solutes to guard cells have been characterized (see Figure 12):

1. The uptake of K^+ and Cl^- coupled to the biosynthesis of malate^{2-}
2. The production of sucrose from starch hydrolysis
3. The production of sucrose by photosynthetic carbon fixation in the guard cell chloroplast
4. The uptake of apoplastic sucrose generated by mesophyll photosynthesis

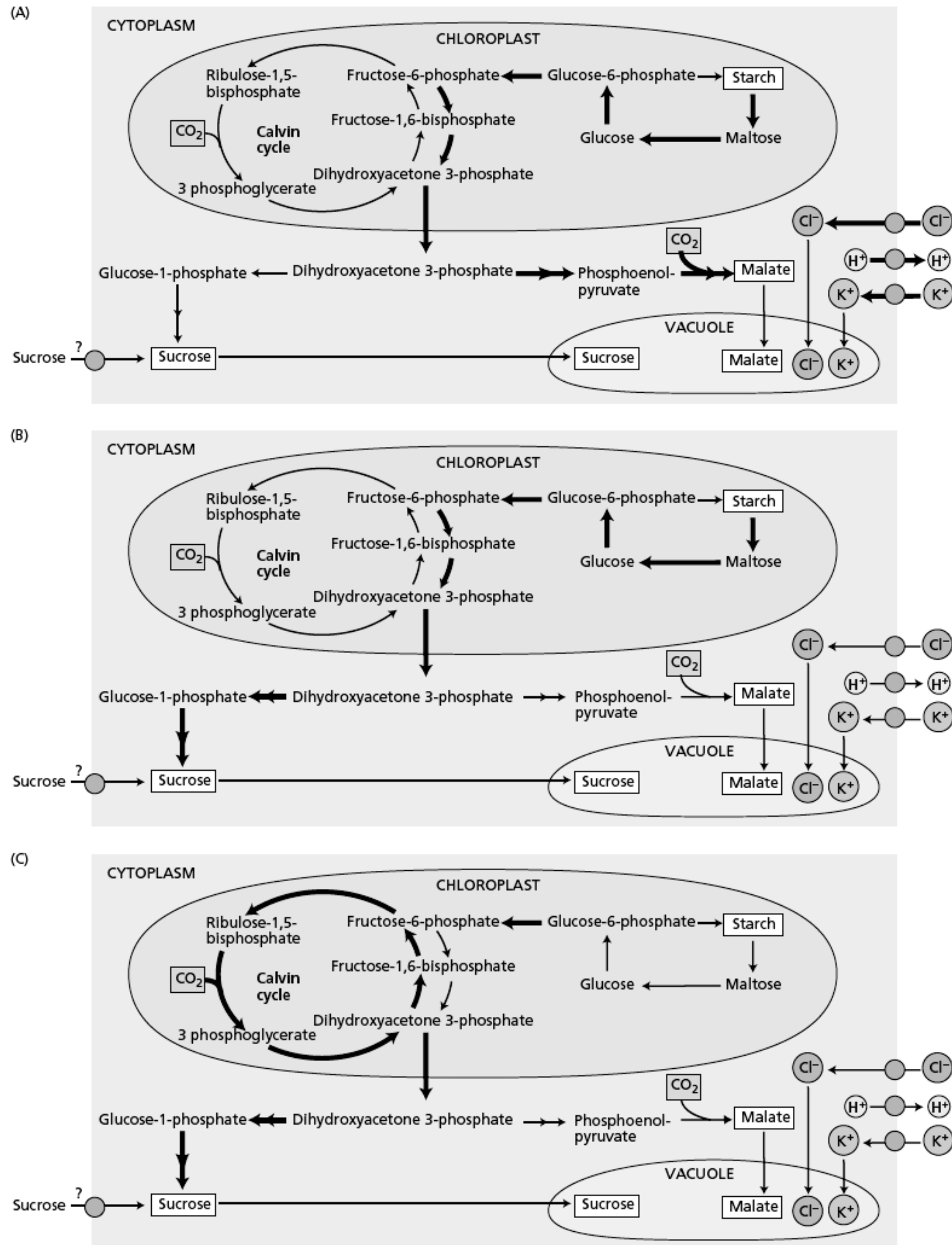


FIGURE 12 **Three distinct osmoregulatory pathways in guard cells. The dark arrows identify the major metabolic steps of each pathway that lead to the accumulation of osmotically active solutes in the guard cells. (A) Potassium and its counterions. Potassium and chloride are taken up in secondary transport processes driven by a proton gradient; malate is formed from the hydrolysis of starch. (B) Accumulation of sucrose from starch hydrolysis. (C) Accumulation of sucrose from photosynthetic carbon fixation. The possible uptake of apoplastic sucrose is also indicated.**

Depending on environmental conditions, one or several pathways may be activated. For instance, red light–stimulated stomatal opening in detached epidermis depends solely on sucrose generated by guard cell photosynthesis, with no detectable K^+ uptake. The other osmoregulatory pathways can be selectively activated under different experimental conditions. Current studies are beginning to unravel the mysteries of guard cell osmoregulation in the intact leaf.

F. Solute transport and photoassimilate translocation: Uptake, transport and translocation of water, ions, solutes and macromolecules from soil, through cells, across membranes, through xylem and phloem; transpiration; mechanisms of loading and unloading of photoassimilates.

1. Water relations

1.1 Introduction

Liquid water is absolutely necessary for life as we know it. Firstly it is the solvent and reaction medium of all living cells, which contain some 75–90% water by weight; secondly it is a reactant in many metabolic processes; and thirdly, as the hydration water of macromolecules, it forms part of the structure of protoplasm, existing as 'liquid ice' in a labile but ordered structure. The physicochemical properties of water (H_2 O) are unique; heavy water (D_2O or DHO), containing deuterium, the heavy isotope of hydrogen, differs sufficiently to be toxic. In multicellular organisms, water provides the transport medium. Additionally, for plants, water is one of the raw materials for photosynthesis and produces the turgor pressure of water-filled vacuoles which gives mechanical rigidity to thin-walled tissues, while some movements of plant organs occur as a result of turgor pressure changes. Plant cell expansion is driven by turgor pressure and hence growth rates depend on hydration levels.

On 'dry' land, the highly hydrated body of a terrestrial plant in many situations tends to lose water to the environment, especially to the atmosphere, in accordance with gradients of free energy of water. There are few habitats where plants do not suffer some water shortage at least intermittently. The necessity for maintaining an adequate internal water content has been a major factor in the evolution of land plants with respect to structure and numerous aspects of physiology. It is not an exaggeration to say that the colonization of land by plants has depended upon the evolution of systems for the absorption and conservation of water. Another difficulty for land plants is the transport of water from the underground supply tapped by the roots to the aerial shoots. For the tallest flowering plants this may mean moving water some 100 m against gravity.

This chapter deals with the forces and factors involved in water uptake and loss in flowering plants, the mechanisms of water movement within the plants, and the controls exerted on water exchange and water transport by the plant and the environment respectively.

1.2 Water movement and energy: the concept of water potential

Water moves into plants, in the case of terrestrial plants mainly from the soil; and water moves out of plants, mainly into the atmosphere. There is also much movement of water within plants. Movement implies the involvement of energy. Metabolism is driven by changes in free energy. Water movement, too, is driven by energy levels. Water will move from a system or area where it is at a higher free energy, to a system where it is at a lower free energy. If we consider a plant cell and its environment, we can therefore say:

When the free energy of cell water is lower than the free energy of external water, net flux will be into the cell.

When the free energy of cell water is higher than the free energy of external water, net flux will be out of the cell.

When the free energy of water is equal inside and outside, there will be no net movement in or out, and if water movement results in equalization of the energy levels, net movement will cease. In both cases, however, a flux (exchange) of water may still proceed, equal quantities moving in each direction per unit time. (This can be demonstrated by applying radiolabelling to the water.)

In order to predict the direction of movement of water into/out of plants, plant cells or tissues, we therefore need a measure for the free energy of water. This measure is the water potential,denoted by the Greek letter C (psi), or Cw. Water moves along gradients of water potential, from higher to lower water potential. Although Cw is basically a measure of free energy, for plant physiology it is most often expressed in pressure units, since hydrostatic pressures and tensions (negative pressures) contribute to water potential and play a very important part in the water relations of plants.

The pressure unit is the pascal Pa and its multiples, the pascal being rather small. Throughout this text, the megapascal MPa (10^6 Pa) is used. For the derivation of water potential from basic principles. It should be noted that water potential applies to water in any situation, and in any form, liquid, ice, or water vapour. Wherever there is water, it has a water potential.

1.3 Water potentials of plant cells and tissues

1.3 .1 Forces determining cellular water potential

The water potential of a plant cell is determined by three kinds of forces which affect the free energy of the cellular water.

(1) In plant cells, the cell wall exerts a hydrostatic pressure, the turgor pressure (wall pressure) on the protoplast; a cell within a compact tissue may also be under pressure from surrounding cells. Hydrostatic pressure in excess of atmospheric increases the free energy and raises water potential; thus the pressure potential ψ_P is a positive value.

(2) Plant cells contain low-molecular-weight solutes, mainly vacuolar in a vacuolated cell. These exert osmotic forces, which decrease the free energy and lower the water potential; the osmotic potential ψ_π is therefore a negative value. (Osmotic pressure is numerically equal to osmotic potential, but has a positive sign.)

(3) Plant cells contain high-molecular-weight colloids, in the cytoplasm and the cell wall. Matric forces exerted by colloids decrease the free energy of water and lower the

water potential; their effect is represented by the matric potential Cm. Surface tension forces at air/water interfaces in cell wall capillary spaces also contribute to ψ_m. The overall water potential of a plant cell is the sum of these three quantities:

$$\Psi = \Psi_p + \Psi_\pi + \Psi_m \quad (1)$$

One can think in terms of the wall pressure tending to squeeze the water out, while osmotic and matric forces tend to draw the water in. In vacuolated cells of high water content, the matric potential is thought to make a relatively minor contribution and, for such cells, the water potential is often given simply as

$$\Psi = \Psi_p + \Psi_\pi \quad (2)$$

But matric potential is important in controlling water uptake and retention by tissues of low water content, such as 'dry' and partly imbibed seeds and in the soil. In the soil, colloid content also can be high.

Osmosis of water is water movement driven by an osmotic potential gradient through a semipermeable membrane, i.e. a membrane permeable to water but impermeable, or very much less permeable, to solutes that are present. Osmosis drives water movement from a lower to a higher solute concentration: a high solute concentration gives a low osmotic potential, hence a low ψ . All membranes of living cells are semipermeable towards nearly all the solutes within the cell, or encountered in the environment, hence osmosis is important in plant water relations. However, as the preceding discussion shows, osmosis is not the only process involved in plant water relations. Hydrostatic pressure (or tension) can overrule osmotic forces. A cell with a positive ψp is said to be turgid, though the degree of turgidity varies according to water content. As a cell takes up water, its ψ rises and its volume expands as the wall is stretched (Fig. 1). When a cell is fully water-saturated, i.e. the wall can yield no more, the cell can take up no more water even from pure water. Its ψ is zero (= that of pure water, with which it is in equilibrium), the osmotic potential of the cell contents being balanced by the pressure potential:

$$\Psi = 0, \text{ and } -\Psi_\pi = \Psi_p \quad (3)$$

This cell is at full turgidity, at maximal volume, maximal wall pressure and maximal water potential – of 0 MPa! The values of plant cell C usually vary from 0 down, i.e. are negative, and negative values are less than zero.

On the other hand, as water is progressively lost from a cell, its ψ decreases, both as a result of the reduction of the pressure, as the shrinking cell contents press less strongly on the wall, and as a consequence of the solutes being concentrated into a smaller volume and lowering the ψπ component. When the stage is reached where the protoplast no longer presses against the wall, the cell is said to be flaccid; now

$$\Psi_p = 0, \text{ and } \Psi = \Psi_\pi \quad (4)$$

If still more water is removed, the effect depends on the mode of removal (Fig. 1).

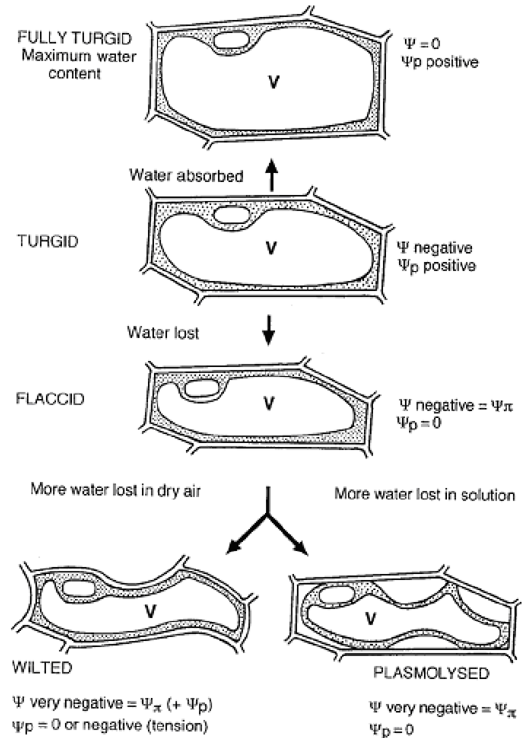


Fig: 1 Effect of water absorption and water removal on a plant cell. Starting with a turgid cell (second from top), absorption of water leads to increase in turgidity and C increases up to a maximum of 0. Water loss beyond a certain level in a solution of lowCresults in plasmolysis, shrinkage of cell contents from the wall. Water loss in dry air results in wilting and the pull of the shrinking contents on the wall can lead to a wall tension instead of a pressure. V = vacuole.

If the water is removed by evaporation, drying out in air, the cell shrivels in size and the wall caves in or folds as the shrinking protoplast pulls on it. The degree of flexibility of the wall may determine how much water can be removed. The tissue becomes wilted. If, however, the water is removed by immersion of the cell in a solution of low ψπ, plasmolysis results: the protoplast shrinks away from the wall and the external solution fills the space between the plasma membrane and the wall; there is no further decrease in overall cell size. The relationships between ψ, ψπ and ψp are shown graphically in Fig. 2 . Under field conditions, wilting is more usual than plasmolysis. Loss of water beyond a limit, which varies with the tissue, is fatal.

Actual values of cell ψ of plants growing in the field in a temperate climate and with a fairly adequate supply of water fall mostly in the range of –0.1 to –2.0 MPa, but may fall well below this in times of water shortage, and in extreme climates or habitats such as deserts or salt

marshes; in the latter the apparently generous external water supply is at a low ψ owing to the osmotic effect of the salt. Plant ψ values below –10 MPa have been recorded.

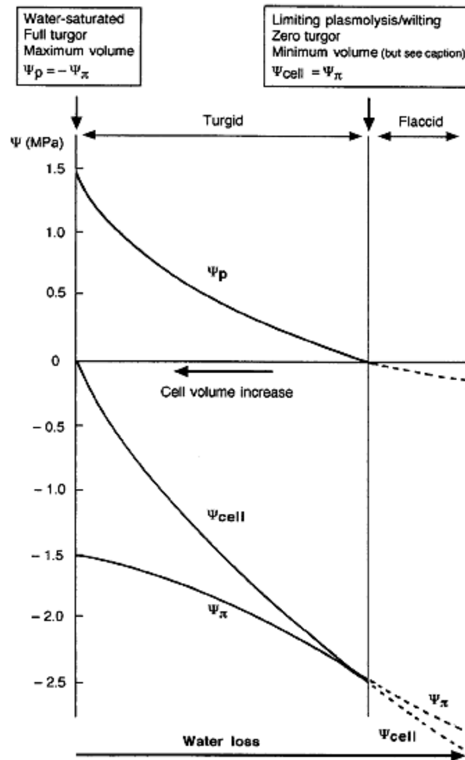


Fig: 2 Generalized quantitative relationship between overall cell Ψ Ψ_π Ψ_p and cell volume. Note that the x-axis scale runs from a maximum value for the volume on the left, at full turgor, to a minimum value at the point of limiting plasmolysis/ wilting. The extent to which the cell volume varies between these points depends greatly on the extensibility of the cell wall; hence no numerical values are given, but if the volume at full turgidity is taken as 100%, the minimum values quoted for different cells vary from about 95% to 70%. The dashed lines indicate what happens if the cell wall caves in under tension after limiting wilting: the volume decreases still further, Ψ_p becomes negative (top section of graph), and the cell Ψ falls below Ψ_π (bottom section of graph). If the wall does not cave in, but the cell wrinkles, Ψ continues to equal Ψ_π with further water loss.

Quantitative consideration of plant water potentials needs clear thinking. Because values of ψ of plants and in the natural environment are negative, one must be careful to keep in mind that e.g. –2 MPa is lower than –1 MPa. Sometimes the terms 'higher' and 'lower' are avoided by referring to 'less negative' and 'more negative' values. One must also accustom oneself to thinking of zero as a high value, the highest that the ψ of plant or soil in most cases can attain. (Plant tissues under high pressure, such as squirting glands or squirting fruits, which can eject their contents to considerable distances, may have positive ψ values. But these play no significant part in overall plant water relations.)

1.3.2 Measurement of water potential and its components in plant cells and tissues

The overall ψ of a cell or tissue can be measured by exposing replicate samples of the tissue to a graded series of ψ, either by immersing the samples in solutions of known ψ, or by enclosing them in atmospheres of known ψ (vapour pressure). Changes in water content of the samples are detected by weighing the samples before and after the incubation period; the tissue ψ equals that of the environment in which it neither gains nor loses water. Another method for measuring tissue water potential is the thermocouple psychrometer. The tissue is allowed to equilibrate with the atmosphere in a small chamber which houses a thermocouple junction and which is incubated at constant temperature. The ψ of the chamber atmosphere will become equal to that of the tissue. A small drop of pure water is then introduced on to the thermocouple junction. As this water evaporates, it causes cooling of the thermocouple, causing a current to flow. The current is proportional to the rate of cooling; the rate of cooling depends on the rate of evaporation, which in turn depends on the ψ of the vapour in the chamber – and this equals the ψ of the tissue. The instrument is calibrated with material of known ψ, so that the tissue ψ can be obtained from the current reading.

The **pressure bomb method** for obtaining water potentials, which is more controversial, is discussed later in connection with xylem transport . It may also be relevant to measure the individual components of ψ. The osmotic potential can be obtained by finding the point of limiting plasmolysis, when the ψp has been just reduced to 0 and the cell's ψ equals its ψπ (Equation 4 and Fig. 2). This is done by immersing the plant material samples in a series of graded ψ. Since the precise point at which a cell just becomes flaccid is in practice almost impossible to see, the point is found at which 50% of the cells can be seen to be plasmolysed, i.e. have gone beyond the limiting point, while the remainder still appear turgid. This point is taken as equivalent to an average limiting plasmolysis value for all the cells. The method does have an inherent error. By the time the point of limiting plasmolysis is reached, the cell volume has shrunk somewhat because of the loss of water and cellular solutes have become more concentrated, lowering the value of the ψπ. Fortunately the volume change is small and the error is estimated at less than 10 %. An alternative method for obtaining the ψπ is to express the cell sap and to measure its ψπ by physicochemical methods applicable to any solution. Errors can be introduced here through dilution of the sap with water from the cell walls, and by chemical changes resulting from the mixing of the vacuolar contents with the cytoplasm.

The pressure potential is more difficult to measure and is often taken as the difference between the overall ψ and the ψπ. However, there is an instrument, the pressure probe, which does measure pressures directly. It consists of a very fine hollow glass capillary needle, oil- or water-filled, which is inserted into the cell and at the other end joined to a pressure sensor. The operation is followed under a microscope, and requires skill. It may be difficult to see precisely where the probe tip is positioned and not all types of cell are amenable to this method.

1.3 .3 Water permeability of plant membranes

Gradients of ψ determine the direction of water movement between cell and environment, and between cell and cell. The speed of water movement into or out of living cells is controlled by membrane permeability. In plant cells, two membranes need to be considered: the plasma membrane (plasmalemma) which surrounds the cell; and the tonoplast, the membrane surrounding the vacuole, which contains the bulk of the water in most mature plant cells. The permeability of biological membranes towards water is generally high. The permeability of plant cell plasma membranes towards water has been estimated to be from several hundred to several thousand times higher than for such small uncharged organic compounds as urea, glycerol or sugars, and for charged ions the difference is greater still. Nevertheless, the resistance of biological membranes towards water is not negligible. The quantitative measure of the permeability of a membrane towards a chemical is the permeability coefficient, the rate of diffusion per unit area, unit time and unit concentration gradient. The permeability coefficients of biological membranes towards water are so high that they are difficult to measure; the concentration gradients are small and equilibration between external and internal water is reached rapidly. Very variable values have been reported for the permeability coefficients of plant cell plasma membranes. Because of the practical problems in measuring the permeability coefficient for water, the hydraulic conductivity is often measured instead, the rate of diffusion of water per unit area and unit time per unit hydrostatic pressure gradient. This can, however, be used only to compare water permeabilities of various systems, not for comparing the permeabilities of water and other chemicals.

The ease with which water, and other small hydrophilic molecules with masses not above 46 Da, traverse biological membranes was first attributed to minute pores opening up transiently in the bimolecular lipid leaflet of the membrane by the random thermal vibrations of the lipid molecules. Subsequently membrane proteins named aquaporins, which make water-specific channels through biological membranes, have been identified in all types of organisms. Plants contain a greater variety of aquaporins than organisms from other kingdoms; over 30 aquaporins are known from maize, for instance. Aquaporins show specificity with respect to organ and membrane, i.e. plasma membrane or tonoplast. Evidence is accumulating that the variability of the reported values of the water permeability coefficients of different plant cells results at least partly from differences in the concentrations (and may be types) of aquaporins. Development and differentiation are associated with changes in aquaporin density; e.g. during the cell elongation stage which is accompanied by rapid water uptake, aquaporins are particularly abundant. A diurnal rhythm in expression of aquaporin genes has been correlated with leaf movements which depend on turgor changes in their motor cells, mediated by water movements in and out of the cells. It must be emphasized that all water movement into or out of plant cells is along the C gradient. There is no active pumping of water against its free energy gradient, at the expense of metabolic energy, as occurs with many other metabolites. The permeabilities of the plasma membrane and tonoplast towards water are so high that the water would leak out very fast; the amount of metabolic energy needed to pump it against the leakage would be unrealistic.

1.4 Water relations of whole plants and organs

The water relations of a whole plant, or even an organ such as a leaf, are much more complex than those of individual cells. The formulae given in the preceding section, relating water potential to pressure and osmotic (and matric) potentials are applicable only at the cellular level. There is no such thing as the ψp of a whole plant; the value will vary between different tissues. The overall ψ, too, usually varies between different parts of a plant. Most flowering plants are land plants and, in the terrestrial environment, the ψ of the atmosphere is nearly always much lower than that of plant tissues, often by tens of MPa, and hence there is a great tendency for water loss from the plant. The large surface area necessitated by the photosynthetic mode of life provides a large surface for evaporation of water, transpiration. This loss must be made good from the soil, which is generally at a much higher ψ than the atmosphere or the plant. Hence for most of the time there is a flow of water through the plant, along a ψ gradient, as below:

soil → root→stem→leaf→air

This flow is frequently termed the transpiration stream. Only in times of a water-saturated atmosphere, or in times of extreme drought, may there be equilibrium, more or less, between plants and the environment, with gradients within the plant eliminated and water movement nearly at a standstill. Of the water absorbed by the roots, only a very small fraction is retained by the plant in temperate habitats. For maize, an annual, this fraction has been estimated at less than 1% of the water absorbed during its growing season. During one bright sunny day leaves may transpire several times their own weight of water; a leaf of *Senecio jacobaea* growing on a sand dune can transpire its own weight in water in 45 minutes. The water content of aerial organs of a plant is generally lower in the daytime, when the rate of transpiration is high, than during the night, when the transpiration rate is much lower (owing to the lower temperature and closure of stomata) and the deficit is made good. In the roots, which experience much less temperature change over the diurnal cycle and which have no stomata, the water content fluctuates much less.

1.4 .1 Absorption of water by roots

The root systems of plants are often very extensive. Roots of some plants extend much further underground than the shoot rises into the air. The roots of apple tree (*Malus domestica*) may go down to about 10 m, and even in herbaceous plants such depths can be reached, e.g. in alfalfa (*Medicago sativa*). The actual area of a root system is formidable. It has been reported that a rye (Secale cereale) plant had a root surface area of over 600m^2, of which two-thirds was root hair area, and the total length of the root system was over 11000 km, including 10000 km of root hairs. The total area of the shoot (including areas of cells bordering leaf air spaces) was only 28 m^2 . Most of the water absorption takes place near the root tips, where

there is a thin epidermis with root hairs. Not only do the root hairs provide a large area, but they make intimate contact with the soil, bending around soil particles and penetrating into tiny crevices.

As the root tissues mature, the epidermis with its hairs is replaced by a more impermeable suberized periderm. For efficient water uptake, root growth must continuously regenerate the absorbing zone behind each growing apex. Continuous growth is also necessary to invade new areas of soil, for there is little lateral movement of water in soil compared with downward drainage directly after water addition. Water will not move to the roots, so roots must grow to the water. Positive hydrotropism may direct root growth towards water. Roots can grow rapidly; a rate of 10 mm per day is common for grasses; maize (*Zea mays*) roots can extend by as much as 50–60mm per day. The average daily increase in length of the total root system of the rye plant discussed above (hairs excluded) was almost 5 km.

Though the root hair zones provide the most efficient water absorbing surfaces, uptake in older regions is still appreciable, particularly during conditions of water shortage and at times when root growth is slow, such as in winter. Points of emergence of lateral roots break the suberized layers and enable the entry of water.

1.4 .2 The route of water movement through the plant

The xylem as the water-transporting system

The main channel for upward/long-distance movement of water in the plant is the xylem, the wood. When the tissues outside the xylem are peeled off over a short length of woody stem where the xylem is central, the conduction of water beyond the stripped region continues unimpeded. The non-living cells of the xylem are filled with a watery sap, at least in young wood, and dyes and Indian ink can be seen to move in the xylem. Toxic solutions have been shown to pass from roots to leaves, indicating that the route does not involve living cells; heat-killed stems, too, can conduct water. Chilling does not stop water movement as long as no freezing occurs. All this points to nonliving cells of the xylem as the water-conducting cells. When the lumina of these cells are blocked with mercury or cocoa butter, water movement is inhibited.

The xylem is a complex tissue. In addition to water-conducting cells and lignified fibres which all are dead at maturity, it contains living parenchyma cells and sometimes also living transfer cells. Functional xylem is accordingly not a dead tissue, though it contains a large proportion of dead cells. In flowering plants there are two kinds of conducting cells, the tracheids and the vessel elements. They have lignified secondary walls with the secondary thickening laid down in distinctive patterns leaving areas of primary wall as pits; these facilitate the passage of water from cell to cell. Somewhat confusingly, the thin primary wall across the pit is often called the pit membrane. The tracheids function as single cells, but vessel elements are joined to make elongate vessels by the perforation or partial breakdown of end walls in files of cells; these end walls are then known as perforation plates. Their possession is the distinguishing feature of the vessel element. The diameters of vessel units range from below 10 mm to several hundred mm, even 1000 mm in some lianas. Tracheid diameters overlap with those of the narrower vessel elements. Any one piece of xylem has conducting elements of varied width; this may be of functional importance. The lengths of vessels range from under 1cm to 10 m or more, and are very variable even within the same plant. In some trees some continuous vessels run right from the crown to the roots, but most vessels are shorter than the height of the plant, and even in trees many vessels measure only a few centimetres. The possession of vessels making long continuous channels for water movement is considered to be one of the advanced features of flowering plants; the earliest land plants had only tracheids, and while the evolution of vessels has occurred in several divisions of land plants they are lacking in the conifers. Tracheids offer much more resistance than vessels to water movement.

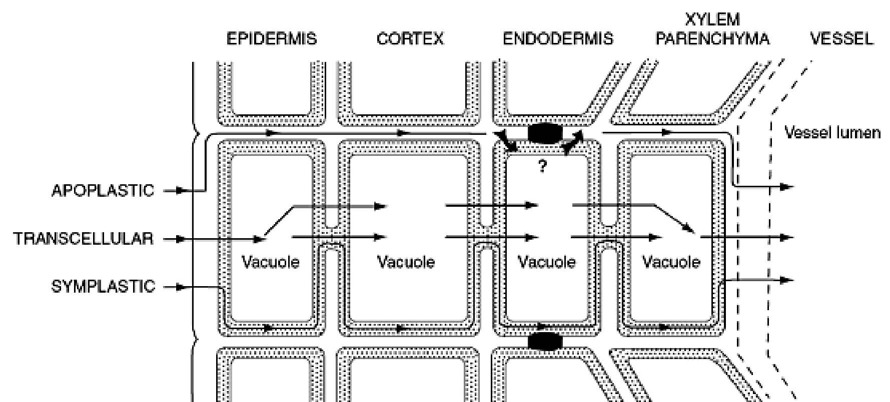


Fig. 4 Diagram of the postulated apoplastic, symplastic and transcellular routes of water movement through the root to the xylem. The arrowed lines represent the pathway of water; the symplast (cytoplasm and plasmodesmata) is stippled; the apoplast is white and the Casparian strips of the endodermal wall are shown in black. The xylem vessel wall and lumen are both parts of the apoplast, but their outlines are shown with dashed lines. For simplicity, all but one cortical cell layer and the pericycle (a parenchymatous layer inside the endodermis) are omitted. The transcellular route is shown in two variations; in the strictest sense it crosses every plasma membrane and tonoplast (upper arrows) but could also combine passage through membranes with passage through plasmodesmata (lower arrows). It is not certain to what extent the water in the apoplastic route has to bypass the Casparian strips as indicated by the curved arrows:

In woody perennials, new layers of xylem known as annual rings are produced each year during the growing season. The older regions of xylem eventually lose their water-conducting capability and become air-filled or blocked by ingrowths (tyloses) from adjacent living parenchyma cells, or by gums, resins and tannins. The water then moves only through the young, outer xylem, the sapwood, which may comprise only the current year's growth or include a few youngest annual rings. The inner non-conducting xylem is known as the heartwood.

Living parenchyma cells make up rays in secondary wood, running radially through the xylem from the pith towards the cortex (Fig. 3) and lying also among the conducting cells. They store organic nutrients, and some botanists have assigned a role in water transport to them. Transfer cells are not always present in xylem, but may occur next to conducting cells especially in leaf veins; they have highly involuted cell walls, which gives them a large surface area of plasma membrane, and they function in

the transfer of solutes into and out of the conducting cells. The movement of water into and out of the xylem

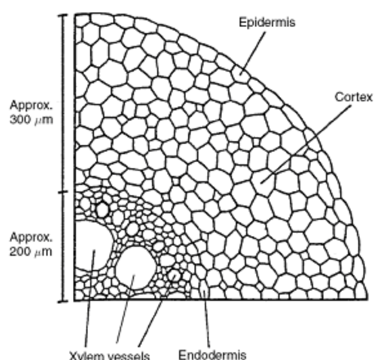


Fig: 3 Transverse section of a portion of a young maize root (Zea mays), showing the tissues through which water must pass on its way from the soil to the xylem. The epidermis, endodermis and pericycle (to the inside of the endodermis) are single cell layers, but the number of cell layers in the cortex varies widely according to species and type of root.

In the root, the xylem is the central tissue; to reach it, water must pass radially through the epidermis, cortex, endodermis and pericycle (Fig. 3). The precise radial movement pathway is still under discussion. There are three possible routes for the water: the apoplastic route, the symplastic route and the transcellular route (Fig. 4).

The apoplast is the collective term for all the non-living parts of the plant body: cell walls, intercellular spaces and xylem conducting cells. Apoplastic water movement would occur in the capillary spaces of the root cell walls and perhaps in the intercellular spaces; there are reports of these spaces in roots containing fluid. The symplast is the collective living part of the plant; nearly all the living cells of the plant body are joined by plasmodesmata, submicroscopic protoplasmic connections of diameters around 50 nm. In the symplastic route, water has to cross a plasma membrane to enter the cytoplasm of an outer root cell; it would then move in the cells within the cytoplasm, around the vacuoles, and from cell to cell through the plasmodesmata without the necessity to cross more membranes, till it exited into a xylem conducting cell. The transcellular route envisages movement 'straight' through the vacuoles, crossing the tonoplasts of each cell; cell-to-cell movement could be via plasmodesmata or crossing the plasma membranes.

The apoplast route has been supported on the grounds that it is the path of least resistance, with minimal traversing of membranes. However, the radial walls of endodermal cells at the level of most active water absorption develop strips of wall thickening, the Casparian strips, chemically resembling the water-impermeable suberin (Fig. 5). In older parts of the root, all endodermal walls except the outer tangential ones become heavily thickened. It is therefore frequently suggested that at least at the endodermis water must pass through living protoplasts, and that this layer regulates water movement to the root xylem. There is some evidence to support this idea, but also data to the contrary, probably reflecting the extent of wall thickening in the material studied. Decreased aquaporin content in plasma membranes of tobacco roots *(Nicotiana tabacum)*, achieved by antisense repression of an aquaporin mRNA synthesis, has decreased greatly the roots' hydraulic conductivity. This indicates that there is movement through the plasma membranes and certainly supports the symplast route. But the data do not exclude the transcellular route. This route has been criticized as being the path of greatest resistance in view of the large number of membranes traversed. However, the density of aquaporins in tonoplasts is very high (up to 40% of total tonoplast protein), which may give them a much higher permeability towards water than is shown by the plasma membranes, and crossing the vacuole may offer less resistance than originally supposed. The three routes are not mutually exclusive and it is quite possible that all three contribute to water movement in proportions varying according to circumstances. When the rate of water movement is slow, most of the movement might be along the low-resistance apoplastic route, the higher resistance pathways beginning to contribute when demand increases. But the opposite has also been suggested, with a strong transpiration pull increasing the apoplastic flow. In a herbaceous plant, where the vascular strands of the stem have not been joined to a continuous vascular cylinder by secondary growth, any part of the root system normally supplies those parts of the shoot which are directly above it, these being the parts with which it is in direct vascular connection; lateral movement does not occur or is very restricted. But if a part of the root system is deprived of water, lateral movement is activated and the overlying aerial parts receive a water supply from other root sectors. In trees with a continuous cylinder of wood, dye injection has shown that the path of water movement frequently spirals round the stem, following a helical arrangement of the conducting cells around the trunk. The route of water movement through stem and leaf tissue after its exit from the xylem is also problematic. Fluorescent dyes introduced into the xylem to act as tracers for water movement move from the conducting cells into cell walls or crystallize out in intercellular spaces in the shoot. Such observations have been interpreted as indicating an apoplastic route for water passing out of the xylem. However, careful analysis of the data points to the opposite view: the dye becomes concentrated in the apoplast precisely because the water passes into the living cells near the xylem, leaving behind the dye to which the plasma membranes are impermeable. It is therefore likely that water in leaves and stems first moves symplastically from the xylem, before it finally passes into cell walls again and evaporates.

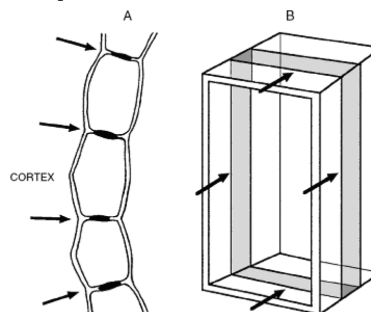


Fig. 5

1.4.3 The motive forces for water movement: root pressure and transpiration pull

The rate of water movement through plants is very variable; Table 1gives examples of maximum rates attained in a number of types of flowering plants, and conifers for comparison. In any individual plant the rate is of course also highly variable, depending on environmental conditions. The order of increasing maximal speed of water movement is the order of anatomical development towards wider and more numerous vessels in the xylem. Conifers, with the lowest maximal speeds, lack vessels, having only tracheids, while lianas may have very wide vessel elements. Water moves through the xylem along a ψ gradient, from roots to all parts of the shoot, most of the flow terminating in the leaves. There must be some motive force, some energy input, which maintains the ψ gradient and overcomes the frictional and gravitational resistances along the way. There are two possibilities: the xylem sap could be pumped up under pressure, or it could be pulled up under tension, negative pressure. Capillary rise (a surface tension phenomenon) could not account for a rise of more than 1 m in the finest conducting elements; many plants are much taller than this, trees reaching 90–100 m.

Table 1 The midday maximum speeds of the transpiration stream, measured by observation of dye movement or heat conduction. Sclerophylls are plants with tough, woody leaves; diffuse-porous wood has large vessels evenly scattered throughout the secondary xylem; in ring-porous wood the large vessels are concentrated into bands..

Plant type	Speed ($m\ h^{-1}$)
Evergreen conifers	1.2
Mediterranean sclerophylls	0.4–1.5
Deciduous diffuse-porous trees	1–6
Deciduous ring-porous trees	4–44
Herbaceous plants	10–60
Lianas	150

Root pressure

In certain circumstances, the xylem sap is under positive hydrostatic pressure; when the plant is decapitated just above root level, the stump exudes sap, a phenomenon often called 'bleeding'. A manometer fitted over such a bleeding stump registers a pressure known as the root pressure, usually in the range of 0.1 to 0.2 MPa, exceptionally reaching 0.5 to 0.6 MPa. The development of this pressure is dependent on the metabolic activity of the roots. No positive root pressure is found when the roots are subjected to treatments inhibiting metabolic activity, such as lack of oxygen, application of respiratory inhibitors, low temperature, or starvation. The mechanism is thought to be osmotic. The exuded sap has a ψ value below that of the soil because of a higher concentration of solutes, mainly inorganic ions, but sometimes including organic solutes, too. It is postulated that the living parenchyma and transfer cells of the xylem secrete the solutes into the conducting cells using respiratory energy. The lowering of ψ causes water to follow the ions into the xylem, building up a pressure – the classical osmotic pressure – which pushes up the sap. The xylem ψ does not equilibrate with that of the soil, since the ions are continuously swept away with the water movement, and the root cells continuously secrete more. In favour of this hypothesis is the fact that when the ψ of the medium around the roots is suddenly lowered, reversing the gradient, exudation rate falls and may even become negative, so that externally applied liquid is sucked in at the cut stump. Root pressure can result in guttation, drops of liquid appearing at leaf tips and edges where the xylem sap is forced out through pores overlying vein endings. This liquid is much more dilute than bleeding sap at a stump, solutes having been absorbed by leaf cells. The manifestation of root pressures in temperate climates is most frequent during warm humid weather and the pressure shows a diurnal rhythm with maxima at nights, often dropping to near zero by day. The drops of guttation fluid are easily mistaken for dew in the morning, but an attentive examination shows that the drops are arranged regularly, corresponding to the positions of the pores. In a strawberry (*Fragaria ananassa*) leaf, for instance, there is a neat droplet at the tip of every tooth of the leaf edge. In tropical rainforests, where it is warm and humid all the time, guttation fluid drips from shrubs and small trees, mimicking rain.

Although the development of root pressures is well authenticated, in the majority of cases root pressure cannot account for water movement. Some species apparently never develop root pressure. The observed pressures are too low to raise water to the required level in tall plants. A pressure of 0.2 MPa can raise water maximally to 10 m, so root pressures in the usual range could raise water no higher than 10 m. The maximal values of 0.6 MPa could raise water to 30 m, still far short of the height of many trees. The quantity of water that can be moved by root pressure is small: e.g. wheat seedlings (*Triticum aestivum*), which transpired about 3 mL water per hour, exuded only 0.5 mL per hour by root pressure. In many instances maximum bleeding rates are only 1–2 % of the water loss occurring by transpiration. Root pressure persists only as long as the water-yielding capacity of the soil is high, but plants can still extract and transport water after root pressure becomes inactivated through a lowering of the soil ψ. As stated, root pressures in temperate climates are most frequently developed during warm nights; most of water transport occurs during daytime. It is moreover mostly herbaceous species that develop root pressures. In deciduous trees root pressures are demonstrable in the spring before the buds open, but once the leaves have expanded and rapid water movement through the plant begins, root pressures can no longer be detected. During the periods of rapid water movement associated with rapid transpiration, the vast majority of evidence in fact indicates that the water is not under positive pressure but under tension. In such circumstances, a cut in the xylem does not result in sap exudation, but if the cut is made under water, the water is sucked in (as seen if a dye is added to the water). Transpiring twigs can pull water against an artificial resistance more effectively than a vacuum pump; leafy twigs can raise a column of mercury to heights greater than can be supported by atmospheric pressure. On the basis of such evidence it is generally accepted that water movement in plants, particularly in woody species, is the result of water being pulled up to replace that lost by transpiration. We therefore need to explain how water can be pulled up under tension to the topmost leaves of the tallest trees.

The cohesion–tension theory (transpiration–cohesion theory) for the ascent of xylem sap

A theory of the ascent of sap based on the cohesive properties of water was advanced independently in 1894 by Dixon and Joly, and in 1895 by Askenasy. This theory, as described below, is still supported in its essentials by most plant physiologists, although alternatives have also been postulated.

The motive force for root pressure is generated at the root end of the plant. The generation of tension takes place at the leaf end. The living cells of the leaf contain solutes and commonly have a ψ well below 0, say down to –2 MPa under 'average' conditions in a temperate climate. But they are still relatively water-saturated compared with the atmosphere for most of the time in most climates. The ψ of the atmosphere is usually very much lower than that of the leaf cells, say –10 to –50 MPa. To put it another way, the atmospheric vapour pressure is usually very much lower than would be in equilibrium with the leaf cells. There are of course occasions when the atmosphere is very humid and water may condense out as mist or dew. But generally, there being a tremendous drop in ψ between leaf and atmosphere, amounting to tens of megapascals, there is a great tendency for water to evaporate, or transpire from the leaf. The cells in contact with air lose water, mainly into the intercellular air spaces: it should be remembered that most of the leaf surface is inside the leaf. Moreover the external surfaces are covered by a cuticle which strongly impedes the passage of water vapour. As the cells bordering the air spaces lose water, their ψ drops and water moves into them by osmosis from the deeper-seated cells with which they are in contact. These in turn replenish their loss from cells still deeper in the tissue, until water is extracted from the xylem conducting cells, especially at the veinlet endings. The 'pull' on the water is transmitted right down the xylem and the water loss is made good by further uptake of water from the soil by the roots. There is thus envisaged an uninterrupted column of water being pulled under tension from the roots to the leaves. The energy for the movement comes from solar heat, which provides the latent heat of evaporation: transpiration is the one plant process which uses solar energy directly without the intervention of photosynthesis. Lignification gives the xylem conducting cells the strength to endure the tensions without collapsing inwards. This briefly summarizes the transpiration–cohesion theory for the movement of water in a qualitative way. The quantitative aspects are now considered. It was stated that the positive pressure needed to pump water up the xylem by 10m is 0.2 MPa; the negative tension needed to pull it up by 10m is numerically equal but opposite in sign, –0.2 MPa. Of this, –0.1 MPa is needed to counteract the force of gravity and a further –0.1 MPa is needed to overcome frictional forces opposing the flow. If 100m is taken as the height of the tallest tree, the mechanism for xylem transport under tension requires that tensions of at least –2 MPa must be generated by transpiration. That is perfectly feasible. The final evaporation of water takes place from the capillary spaces between cell wall fibrils; these spaces may be as narrow as 5 nm and the tensions generated at the tiny menisci (the curved air–water interfaces) can be calculated to reach –2 9 MPa. There is accordingly no problem with the generation of tensions.

A second requirement for this mechanism of water movement is that the water in the xylem must be able to withstand the necessary tensions without the columns breaking. Here the situation is more complicated. The cohesive power of water is great, resulting from hydrogen bonding between the molecules: theoretical calculations of the tensile strength of pure water have given values as low as –1400 MPa. Tensions of –20 to –30 MPa are claimed to have been withstood experimentally. But xylem sap is not pure water: it contains dissolved materials. Taking into account the presence of these, together with the diameters of xylem cells and the adhesive properties of their walls, the expected tensile strength of xylem sap in situ has been put at about only –3 MPa. This, however, would still be enough to raise the sap to 150 m, higher than the tallest trees.

The experimental measurement of tensions in the xylem is not easy and most workers have relied on indirect estimates. One technique is known as the pressure bomb method of Scholander, who introduced it in the 1960s. Branches or leaves are cut from transpiring plants, where, if the cohesion theory is correct, the xylem fluid should be under tension. The leaf/branch is enclosed in a pressure chamber with the cut end protruding and nitrogen gas at increasing pressures is applied inside the chamber; eventually a liquid droplet is seen appearing at the cut end. The xylem tension before cutting is taken to equal the pressure at this point, only with a minus sign. The argument is that, when the cut is made, the water column in the xylem snaps and the water retreats deep into the xylem strand; to force it back to its original position, a pressure is needed numerically equal to the original tension. With this technique, tensions of – 0.5 to –8.0 MPa have been recorded, the highest values occurring in halophytes and desert plants, which must extract their water against a very low ψ in the soil. Twigs of trees have been centrifuged, the centrifugal force exerting a tension on the xylem water until the water columns broke; according to species, tensions from –0.4 to below –3.5 MPa were endured.

Another indirect method for estimating the xylem tension is to measure the water potentials of living leaf cells in contact with the xylem. In wilted plants of tomato (*Lycopersicon esculentum*), privet (*Ligustrum lucidum*) and cotton (*Gossypium barbadense*), leaf ψ values were found to reach –4.1, –7.0 and –14.3 MPa respectively. It was then assumed (without direct proof) that the xylem sap was under a tension of the same magnitude, since the tension must be balanced by the ψ of the leaf cells if any water is to move into the leaves. If numerical values for xylem tensions of magnitudes as quoted above are accepted, these support the cohesion theory. There have also been measurements showing gradients of tension or leaf water potential, with the values becoming progressively more negative higher up the plant – as is of course required for the flow.

There is nevertheless a serious problemwhich has led to reservations in accepting the transpiration–cohesion theory. Water columns under tension are liable to cavitate (embolize) on mechanical disturbance, breaking up into droplets of water and water vapour. When water is sealed into a glass capillary under tension, the slightest tapping

or shaking will bring about cavitation. Plants are buffeted by the wind, sometimes very violently. Any introduction of an air bubble would similarly break up the column. Twigs, even large branches, are broken off by the wind; animals bite away pieces; any such damage might be expected to let air spread through large expanses of xylem, if not the whole plant. Even without external damage, air seeding, i.e. the sucking in of air through the cell walls, is very likely when the xylem water is under high tension. Yet the plants go on conducting water. The actual great stability of the water-conducting system under natural conditions seems at variance with the metastable state of water columns under tension. Twigs which are cut so as to allow air into the vessels will resumewater uptake when the severed end is placed in water. In the winter xylemsap may freeze; the dissolved air trapped as bubbles in the ice should remain as bubbles when the sap thaws – yet sap flow is resumed in the spring. On the other hand, cavitation does certainly occur in plants. Each cavitation event in the xylem makes a minute noise that can be amplified by means of suitable electronic equipment to an audible ‘click’. These clicks can then be counted and recorded over time. During rapid drying out one can count several hundred clicks per minute in a wilting leaf. When the water supply is restored, cavitation ceases and water uptake is resumed. Another method for detecting cavitation is to freeze plant material very rapidly with liquid nitrogen and then to examine the xylem in the frozen state by cryoscanning electron microscopy. Empty (i.e. embolized) and ice-filled vessels are easily distinguished. Cavitation has turned out to be frequent in the field under natural conditions. These observations are difficult to reconcile with the cohesion theory and some plant physiologists postulate alternative mechanisms.

1.4 .4 Validity of the cohesion–tension theory

Alternative hypotheses

All the experimental values for xylem tensions quoted above in support of the cohesion–tension theory have been obtained by indirect means. It was considered for many years that the direct measurement of tensions in the xylem was not practicable. Since 1990, however, a group of workers led by U. Zimmermann has published measurements which are claimed to be direct readings of pressures within intact, water-conducting xylem using a pressure probe. It is not feasible to insert this delicate glass needle, 10 mm in diameter, into thick wood, but fine veins such as in leaf midribs were probed and the probe failed to register tensions as strong as obtained by the indirect methods, –0.4 MPa being the usual limit. Occasionally down to –0.6 MPa was registered, but then the xylem vessels cavitated within minutes of probe insertion. Moreover, many of the probe readings failed to register any tensions at all but recorded weak positive pressures, up to 0.1 MPa.

On the basis of the pressure probe readings, alternative mechanisms for xylem transport have been put forward. Zimmermann and coworkers suggest that the mass flow of sap in the xylem is aided osmotically (in a manner similar to the building up of root pressures), by the secretion of solutes into the conducting cells, from the living cells of the xylem. There would still occur a transpiration pull, but the magnitude of the tension would be reduced and hence there would be no high tensions and less risk of cavitation. Zimmermann claims that xylem sap is quite rich in solutes, contrary to the generally accepted view that it is extremely dilute (except in early spring, before transpiration commences). Another idea (Canny 1995) is that, whilst transpiration provides the driving force, there is no building up of large tensions because the living turgid cells of the xylem exert a positive pressure on the vessels and tracheids, as does the phloem, where cells are under a high positive pressure. In defence of the cohesion–tension theory The majority of plant physiologists nevertheless support the cohesion– tension theory. The new postulates of Zimmermann and Canny rely on tension measurements from one single type of instrument, the pressure probe, whereas evidence for strong tensions has been obtained with several methods, with consistent results. The discrepancy may be due to problems with handling the pressure probe. Its insertion could cause cracks in the xylem cell walls, letting air in and causing cavitation. The probe diameter of 10 mm is not negligible compared with the diameters of the xylem vessels probed, 50–90mm; the insertion of the probe could do appreciable damage. With living cells, the plastic cell contents pressing against a pierced wall could seal up cracks, but in the xylem conducting cells there is nothing to protect against air entry. Claims for a high solute content could derive from the probe sampling preferentially the young xylem (which is outermost), where immature cells might still contain solutes derived from the breakdown of cell contents; such cells might also be under more positive pressures. Later experiments by other workers have succeeded in measuring stronger tensions in the xylem with the probe, down to –1 MPa, and have shown agreement between pressure-bomb and pressure-probe data. The osmotic theory implies a high energy expenditure by the living xylem parenchyma cells for pumping solutes into the xylem, but the low O_2 level in the xylem of woody axes precludes high aerobic respiration rates and the limited volume of living cells would moreover have to control a large volume of dead conducting cells (Richter 1997). There would also have to be a mechanism for recycling the solutes. Mercury is pulled up by a leafy, transpiring twig inserted into the top of a water-filled glass tubing, which has its bottom end in a reservoir of mercury. The twig sucks the mercury into the xylem and right into very narrow tapering cell tips, which would require a tension of –2 MPa, and there is of course no question of osmotic forces acting on the mercury. Strong positive pressures, postulated to be exerted by living xylem parenchyma and by the living phloem, would probably have little effect on the conducting cells with their rigid walls.

At present, the bulk of the evidence seems to be in favour of the cohesion–tension theory; but then one has to account for the fact that airlocks can be introduced into the xylem in nature by cavitation owing to water stress, freezing, mechanical damage – or experimentally, without permanently or even temporarily stopping the overall flow. How is this to be reconciled with the need of continuous water columns for the movement under cohesion?

To account for the resumption of sap flow in the spring after freezing, the suggestion has been made that the air

bubbles released from thawing ice might be forced into solution again by root pressures, which are highest in early spring, although root pressures tend to be low in trees. Another mechanism by which cavitated vessels could be refilled at any season is by capillary forces. Perhaps refilling of cavitated cells is a situation where the activities of the living cells of the xylem and of the phloem, emphasized by Canny and Zimmermann, do come into play in building up pressures and forcing fluid into the empty vessels. Counts of embolized vessels in sunflower leaves at different times of day, and at various stages of drying out, have revealed far fewer empty vessels than expected from the rate of cavitation. From this it was deduced that the embolized vessels were refilled within minutes by pressure from living cells. A similar conclusion was reached from scoring maize roots from dawn to dusk for embolized vessels. It is also possible that many airlocked conduits never resume water conduction, since in perennials a newring of fresh sapwood is produced annually; in some species, only the current year's growth is active in transport. Redundancy of xylem does occur. The total amount of xylem in a plant is generally much in excess over that needed to satisfy the plant's water requirements, as shownwhen large amounts ofwood are removed. The presence of quite large numbers of cavitated vessels can therefore be tolerated without too much ill effect.

There is also evidence that airlocks produced by cavitation do not necessarily spread from their origins over large volumes of xylem. Vessels are formed by perforation of end walls between individual vessel element cells, but very often a perforation plate is crossed by bars of cell wall material which break up the opening into numerous fine slits. It is suggested that in such vessels an airlock may remain confined to the single vessel element in which it forms, unable to pass the perforation plate due to water menisci holding firm in the narrow openings because of surface tension forces. Even if a whole vessel cavitates, its length may be limited compared with the height of the plant. In trees, some vessels may extend to over 10 m, but most are much shorter; e.g. in a holly (*Ilex verticillata*) the maximum vessel length was 1.3 m, but 99.5% of the vessels were under 5cm long. Similar data can be quoted from other species. Cavitations of limited extent can be bypassed by lateral flow through the cell walls (Fig. 6), mainly through pits. Water movement can continue even in the presence of two overlapping cuts from two sides of a stem, as long as the distance between the two cuts is great enough to leave some intact vessels within this region. An increase in the resistance to flow can be detected when such cuts are made, as would be expected: cell walls offer more resistance to flow than the lumina of vessel elements. But the cohesive system as a whole is not seriously reduced.

The necessity to protect the xylem transport system against the effects of cavitation and airlocks may explain why tracheids have persisted in the flowering plants alongside vessels. The vessels, with no barriers to water flow between cell and cell, offer much less resistance to water flow than tracheids, where water must pass from tracheid to tracheid through the cell wall, mainly through pits.

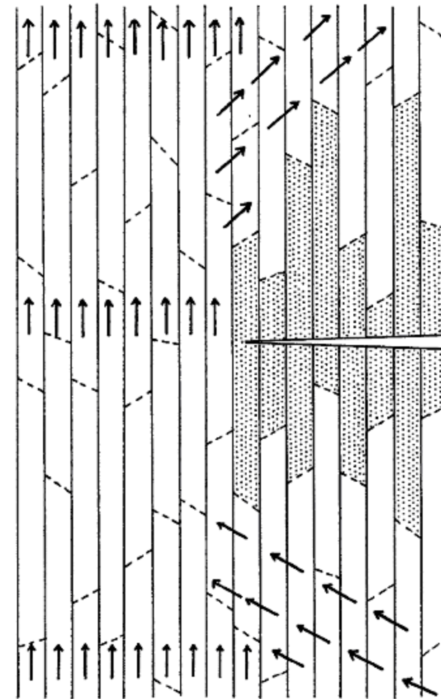


Fig: 6 Bypassing an airlock (embolism) in the xylem following a cut at the right. The slanted dashed lines represent vessel end wall perforation plates; the arrows show the pathways of water flow. Air-filled vessel elements are shaded. The necessity to move sideways through cell walls (via pits) increases the resistance to water movement in the damaged area.

Moreover some vessel elements are extremely narrow, there being normally a range of widths of vessel elements in the same plant organ. Volume flow through a tube is proportional to the fourth power of the radius of the tube. Hence a two-fold increase in the radius of a vessel from say, 5 mm to 10 mm would increase volume flow (for the same tension) 16-fold ($2^4 = 16$). Since wide vessels are so much more efficient for a bulk flow of water, why should a vascular bundle contain, in addition to wide vessels, narrow vessels and tracheids? The most probable answer is that this gives the plant the flexibility to react efficiently to varying environmental water status. When the soil ψ is high the plant does not require very high tensions in the xylem to extract the water; in such a situation, most of the transpiration stream would pass through the widest vessels, which offer the least resistance. But when water stress sets in and xylem tension increases, it will be the widest vessels that are the most vulnerable to cavit ation. The narrower vessels and the tracheids then can take over the function of water conduction; the same high tensions which cause vessels to cavitate also overcome the resistance in the narrower conducting cells. In tracheids, any cavitation event is confined to one single cell, and tracheids are also relatively narrow, so they are the least vulnerable to water stress.

The movement of cohesive water columns in the xylem under transpiration pull may thus be buffered against serious disruption from cavitation by excess capacity; by the regular annual replacement of old xylem by new in perennials; by refilling of air-filled vessels by root

pressures, by capillarity and by pressure from adjacent living cells; by the bypassing of airlocks in cell walls; and by the presence in xylem of tracheids and narrow vessels, which are less susceptible to cavitation. There is not enough evidence for accepting alternative theories for the ascent of sap which deny the existence of high tensions in the xylem. Nevertheless, data from the pressure probe measurements, and other apparently anomalous observations, are drawing attention to the possible functions of the living cells of the xylem, and of the neighbouring phloem, in water movement.

1.5 The transport of solutes in the xylem

Xylem transport is often considered primarily in terms of water movement, but the xylem also has an important function in the transport of solutes. Samples of xylem sap can be obtained for analysis by collecting the exudate when it is under pressure (the bleeding sap); or it can be sucked out under vacuum, pressed out or centrifuged out, from pieces of wood. The concentration of dry matter in xylem sap is low, commonly 0.1 to 0.5%, but the total volume of sap moved in the xylem is high, so the amount of solute that is carried in the xylem is significant. The mineral ions absorbed by the roots are distributed around the plant in the xylem; this can be shown by tracing the pathways of the ions with radioactive labelling, as well as from sap analysis. From two-thirds to three-quarters of the solids are, however, organic, including amino acids, amides and carboxylic acids, giving the sap a pH of about 5. The xylem sap composition in any individual plant varies with the environmental conditions; during rapid transpiration, when large amounts of water are passing through the xylem, the concentration of solids may fall very low. There are also more regular seasonal variations correlated with plant development. In woody perennials, the mineral content of xylem sap is highest in the spring, when active growth is resumed. The carbohydrate content of xylem sap is usually below 0.05% and may be undetectable. But in woody species in the early spring, before leaf expansion and the onset of transpiration, there may be a period of high sugar content in the xylem sap, up to ^{0}%. This sugar is derived from reserve starch, stored in the woody stem over the winter. During this period of sugar mobilization, the xylem sap acquires a positive pressure. The sugary sap flows out in quantity from cuts made into the wood, and the tapping of birch (Betula spp.) and sugar maple (*Acer saccharum*) in the spring has been a tradition in northern regions of Europe and America probably for millennia. Maple syrup is commercially produced from sugar maple sap. Plant hormones can also move in the xylem; e.g. the sensing of water stress in roots stimulates the synthesis of abscisic acid, which is transported in the transpiration stream to leaves, where it induces stomatal closure. Other hormones transported up in the xylem are important in growth correlations between root and shoot.

1.6 Water uptake and loss: control by environmental and plant factors

The rates of water absorption and water loss, and consequently of water movement through the plant, are determined by an interaction between plant and environmental factors. The environmental factors can be classified as soil (edaphic) and atmospheric. With regard to the soil, important considerations are the amount and availability of soil water, soil temperature and soil aeration. Above ground, the relevant factors are atmospheric humidity, temperature, wind speed and light. The plant factors are the area and water permeability of the absorbing surface in the roots; the area and water permeability of the evaporating surfaces of the shoot; the frequency of stomata and the degree of their opening.

1.6.1 Soil water and uptake by the roots

The soil is a complex system. Physically, it consists of particles with sizes ranging from large stones to submicroscopic colloidal material, and it contains pores of varied dimensions. Chemically the particles are of various composition, organic and inorganic, and there are many solutes in the soil water. The high colloidal content of most soils (coarse sand is an exception) gives it a significant matric potential; solutes such as mineral ions give it an osmotic potential. The pressure potential is represented by tensions (negative), i.e. the surface-tension forces at water menisci in small pores. Electrostatic forces around particles and capillary forces in pores also decrease free energy and help to retain water in the soil. When a soil is saturated with water, all the pores are filled, but a well-drained soil does not remain water-saturated for long. Water drains away quickly under gravity from the larger spaces, but some is retained in the smaller pores by the colloidal, surface-tension and capillary forces, and as adsorbed surface films around soil particles. When a soil contains as much water as it can hold against gravity, it is said to be at field capacity. The amount of water present at field capacity depends on the soil type. Soils with fine particles have many small pores and much total particle surface area, and can hold more water than coarse soils. A clay soil at field capacity may hold 55% water on a dry-weight basis (i.e. 55 g water per 100 g dry soil), while a coarse sand may hold only 17%. Once the water content has fallen to field capacity, there is almost no movement of liquid water in the soil, though water evaporates to the atmosphere. TheCof a soil at field capacity is very high, just below zero (unless the soil is highly saline) and uptake by plants can proceed freely. As the water content of a soil falls, its C decreases progressively. The concentration of solutes rises and the Cp falls and the smaller volumes of water between soil particles have more curved menisci; this increases the surface tension forces and lowers the C. Also, as the outer layers of water are removed from the surface films, the inner layers are held more strongly by electric charges and van der Waals forces. At first the lowering of the soil C is matched by lowering of the Cin the plant and water absorption continues. Eventually a stage is reached, however, when the soilCfalls so low that the plant can no longer obtain enough water to compensate for transpirational losses, and it wilts. At first this may be temporary, the plant wilting by day but recovering at night, when the transpiration is low and water uptake catches up with water loss. Eventually there comes the permanent wilting point (PWP), defined as the stage when the plant will not recover from wilting unless more water is added to the soil. Numerically the PWP is expressed as the percentage of water left in the soil. The value of the PWP depends on the species as well as on the soil,

different species reaching the PWP at different values of soil ψ.

Water uptake does not totally cease at the PWP but leaf turgor pressure remains at zero. Once the PWP has been reached, removals of very small amounts of soil water cause very large decreases in the soil ψ: the relationship between soil water content and soil C is not a linear one (Fig. 7). Whether the plant can survive wilting depends on the species, on the degree of water loss and on the length of time in the wilted state.

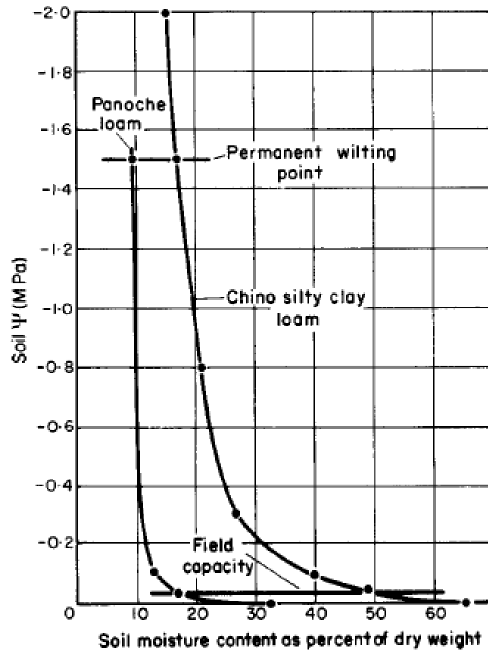


Fig: 7 The relation between soil moisture content and soil Ψ in a sandy soil (Panoche loam) and a clay soil (Chino silty clay loam). The permanent wilting point (PWP) varies between plants, but many species reach PWP at about –1.5 MPa, as indicated in the graph.

Most of the water uptake by a plant takes place when the soil moisture is between field capacity and PWP. As the soil dries out, the forces opposing plant water uptake increase; the rate of water uptake, plant hydration and plant growth rate can be impaired by water stress even if wilting is not reached. This reduction of growth rate can aggravate the effect of water shortage, for the slowing down of root growth decreases the rate at which new areas of the soil are tapped by the roots.

Soil aeration affects water uptake. Field capacity is the ideal state of soil for plants since it has a high C but also has air-filled spaces. A fully water-saturated soil has an even higher C, but it is waterlogged, without air spaces. An adequate O_2 supply is necessary for root growth; a lack of O_2 and a high concentration of CO_2 accumulating from anaerobic respiration of roots and soil microorganisms) are moreover reported to decrease the permeability of roots to water. Soil temperature affects root growth and root permeability, both being decreased at low temperatures. The viscosity of water on the other hand increases as the temperature falls; low soil temperature may thus considerably reduce the uptake of water by roots under transpiration pull, leading to 'physiological drought' when there is water available in the soil, yet the plants suffer water stress. Root pressures develop only in warm, well-aerated soils of favourable moisture content.

1.6.2 The atmosphere and transpiration

The daily course of water absorption by plants closely follows, with a time lag, the course of transpiration. Thus the atmospheric factors which determine the rate of transpiration also largely determine the rate of water uptake.

The rate of transpiration, the outward diffusion of water vapour from plants, is subject to the same physical laws as the inward diffusion of CO_2. Transpiration is directly proportional to the water potential gradient Δψ between the leaf and the air. Or, since atmospheric water status is often expressed in terms of vapour pressure Δe, which is proportional to ψ , we can substitute Δe, the water vapour pressure gradient, for Δψ. Transpiration, on the other hand, is inversely proportional to Ra (boundary layer resistance) and Rs (stomatal resistance). Denoting the rate of transpiration by T, we have:

$$T = \frac{\Delta\Psi}{R_a + R_s} = \frac{\Delta e}{R_a + R_s}$$

There is no term in this equation to correspond with the mesophyll resistance which is involved in CO_2 diffusion, for water vapour does not pass through cells on its way to the outside. Transpiration rate increases with increasing temperature, a rise in temperature resulting in a steeper concentration gradient of water vapour out of the leaf, i.e. Δe increases. The air spaces within the leaf are normally at near saturation vapour pressure, c. 100% RH (relative humidity, the ratio of actual vapour pressure to saturation vapour pressure as a percentage). The absolute concentration of water vapour at a given RH increases with increasing temperature, i.e. air at 100% RH at 20 ^{0}C will contain more water vapour than air at 100% RH at 10^{0}C. A rise in leaf temperature therefore increases the vapour pressure in the leaf without a corresponding rise in the external air. The gradient is approximately doubled for a 10^{0}C rise in temperature. Transpiration decreases with increasing atmospheric humidity, for this decreases the Δe. Wind stimulates transpiration, decreasing Ra as it sweeps away the water vapour accumulating in the boundary layer at the leaf surface. By causing bending of the leaf it may cause mass flow of air into and out of the leaf, thereby enhancing water loss. Light has no direct effect on water loss, but it does have a profound effect on transpiration indirectly: it warms up the tissues, increasing transpiration, and it promotes stomatal opening. The combined effects of light and temperature result in the diurnal changes in the rates of transpiration (and hence of water uptake).

1.6.3 Stomatal control of transpiration

The features of the plant which control the rate of passage of water through it act largely through controlling the rate of transpiration. If comparison is made between plants of

different species, or different individuals of the same species (which can show variation according to their growing conditions), the following shoot characters are seen to favour rapid transpiration: a thin cuticle; lack of hairs; a high stomatal frequency per unit area; a large surface area and a large ratio of internal to external surface area. The water uptake capacity of the roots can also limit the rate; when half the leaves are removed from a plant, the remainder may transpire more rapidly per unit area of leaf, being now able to draw on the whole root system rather than on half of it.

However, when an individual plant is considered over a short term so that developmental changes in, say, the extent of the root system do not come into play, the degree of opening of the stomata is often the most important single plant factor directly controlling the rate of transpiration. By far the greater proportion of the water lost from the leaf comes from the leaf air spaces via the stomata, although the stomatal pore area may be only 1–2 % of the total leaf surface. The daily course of transpiration rate follows closely the course of stomatal opening. Where there is a midday closure of stomata, this is always accompanied by a reduction of transpiration rate. In plants with a thick cuticle, such as the bay laurel (*Laurus nobilis*) water loss through the epidermal surface excluding stomata, the 'cuticular transpiration', may be as low as 2 % of the total transpiration. When the cuticle is thinner, cuticular transpiration can constitute up to about 50% of the total; the cuticular transpiration of the 'average' mesophyte is 10–2 5% of the total. Closing of the stomata will therefore, according to species, reduce transpiration to some 2 –50% of that occurring with fully open stomata.

Transpiration is slowest when stomata are completely closed and increases with increasing stomatal opening. If the atmospheric conditions favour rapid transpiration, the increase continues right up to maximum opening, the stomatal aperture being the limiting factor.

But if the atmospheric conditions do not favour rapid transpiration, maximal transpiration rate may be reached when the stomata are only partly open, the external conditions becoming limiting. In the pathway of water movement from soil to atmosphere, the stomata are situated between the leaf air spaces and the atmosphere:

Soil→Root→Stem→leaf → Stomata→air

That is the point where the drop in ψ is the greatest, and therefore the stomata can exert very effective control over water movement when, in moving air, the boundary layer resistance is low. In still air, however, the boundary layer resistance may become the limiting factor and be more important than the stomatal resistance. Partial stomatal closure cuts down the rate of transpiration more than the rate of CO_2 diffusion; there being no mesophyll resistance for water vapour movement, the stomatal resistance assumes proportionally more importance.

Stomata are highly sensitive to water stress and some species react by (partial) closure at quite low levels of water deficit, protecting the plant against further water loss. In wilted plants the stomata are shut. The early stages of wilting may, however, be accompanied by a widening of the stomatal aperture, the guard cells being pulled apart by the shrinking of surrounding epidermal cells, which lose water more rapidly. In extreme wilting, too, the protective mechanism may break down as the epidermal cells shrink and again pull the pores open.

1.6.4 Waterproofing the surface: cuticle and wax

One of the key features that arose during the evolution of terrestrial plants is the xylem, which makes possible the transport of water to parts of the organism not in contact with a water supply. Another key feature was the evolution of waterproofing chemicals which cut down the rate of evaporation from plant surfaces. The control of water loss by stomata can be significant only if the rest of the surface is waterproofed to some extent. This is achieved by the presence of the cuticle and wax.

The whole outer surface of all land plants (even the Bryophyta) is covered by a cuticle. In the flowering plants, the cuticle covers not just the external surface, but the walls of cells lining internal air spaces, although here it is extremely thin; there is a very thin cuticle on the root epidermis, too. A cuticular ridge commonly overarches stomata. The cuticle is a continuous skin over an organ, not separate for each individual cell; it can be detached by chemical treatment in one piece from e.g. a leaf. The cuticle is made up of several layers which differ in chemical composition, but the main component in all layers is cutin, a complex hydrophobic polymer of mainly hydroxy fatty acids. Some wax (see below) may be present in the cuticle, and secondary compounds such as tannins. The innermost layer of the cuticle is rich in pectins; when the pectins are hydrolysed, the cuticle is detached. The thickness of the cuticle varies from a fraction of a mm to over 10 mm, being thick in plants of dry habitats.

On top of the cuticle aerial organs have a layer of epicuticular wax. This is attached only loosely and can be wiped or rubbed off. The wax gives the 'bloom' to glaucous leaves and fruits; some dark plums and so-called black grapes look almost sky-blue when untouched, owing to the wax, but much of that gets rubbed off when the fruit is handled. The sheen and texture of many floral parts result fromtheir wax layer. The wax is not a pure chemical, but a mixture of long-chain hydrocarbons, long-chain fatty acids, long-chain hydroxy acids, esters, alcohols, aldehydes and terpenoids. Each species produces its own mixture; about 50 different chemicals have been detected in apple wax. Even as for the cuticle, the thickness varies from a fraction of a mmupwards. The wax palm(*Klopstockia cerifera*) native to the Andes, has 5mmof wax on its leaves; carnauba wax, from the leaves of Copernicia cerifera, is harvested commercially in Brazil.

The thickness and chemical composition of the cuticle and wax determine the extent of transpiration with closed stomata, the 'cuticular transpiration'. The more impermeable these layers, the smaller is the cuticular transpiration (and the more complete is the control exerted by the stomata). The cuticle and wax also prevent the entry of water. It would not be a healthy situation if rain penetrated plants freely, flooded the air spaces, soaked the cell walls and leached out solutes from the apoplast. Raindrops largely roll off owing to the hydrophobic nature of the surface. The wettability is determined mainly by the epicuticular wax, its thickness

and even more by its structure. To the eye a waxy leaf or fruit looks smooth. Microscopy, especially scanning electron microscopy, reveals that the wax is present as plates, rods, granules or tufts. These formations increase the hydrophobicity; even smooth wax is water-repellent, but a surface bristling with small wax projections is almost unwettable. Agricultural and horticultural sprays are mixed with detergent to lower the surface tension and enable the fluid to wet plant surfaces. The cuticle and wax also give some protection against the penetration of pathogens, and against ultraviolet radiation. The mode of production of these extracellular layers by the plant is something of a puzzle.

The components are generally assumed to be secreted as liquid precursors by the epidermal cells and to solidify on the outside, otherwise continuous layers cannot be produced, nor can solid wax rods be transported through a cell wall. The most difficult problem is the wax, which must move not only through the cell wall, but through the cuticle as well. It might be suggested that the wax layer is formed first and then the cuticle under it; but when the wax is wiped off, leaving the cuticle intact, a new layer of wax is formed by young leaves (and in some species by mature leaves also). Searches for channels in the epidermal cell walls have not given clear-cut results. There have been reports of channel-like structures in cell walls seen by light microscopy, but these have not been confirmed by electron microscopy. Another idea is that the wax molecules are carried along with water vapour molecules during cuticular transpiration, a process that has been compared to steam distillation.

The patterns of the wax can be produced physically, with no control from the living cells. Waxes from various species dissolved in organic solvents and allowed to crystallize out as the solvent evaporates have been found to crystallize into the pattern originally exhibited on the epidermis. However, in some cases the pattern has differed from the original.

1.6.5 Is transpiration really necessary? To put it another way, could water be moved to the tops of plants in sufficient quantities by any other mechanism, root pressure having been shown to be insufficient?
Continuous columns of water under tension could in fact be maintained as water is used up in photosynthesis, and in any other chemical reactions where it is a reactant. As long as something removes water at the leaf end, the water would be pulled up; it does not have to be removed by evaporation. Osmotic forces in the leaf cells could also maintain ψ gradients between the leaf and the root (and soil). But the amount of water moved in such circumstances would be no more than is used up in growth, in chemical reactions, and in maintaining the hydration levels of the leaf cells. Xylem transport is important also in distributing mineral ions, organic nitrogenous compounds and hormones. The critical question is whether the much slower water movement that would be maintained in the absence of transpiration would suffice for the distribution of these solutes. Information on this is limited. One investigation with sunflower plants indicated that the suppression of transpiration had no adverse effect on mineral ion uptake and distribution over 30 days. But one wonders whether rapid xylem transport might be advantageous for the transmission of hormonal stimuli in situations such as the sensing of drought by the roots.

The cooling effect of transpiration is also very important in keeping down the temperatures of plant organs, especially in hot weather. Throughout the day, a leaf absorbs radiant energy. The amount of energy stored in photosynthate is about 1% of that absorbed, maybe up to 4 % in some C_4 species. The rest of the absorbed energy is converted to heat. If the leaf is not to heat up steadily, this heat must be dissipated, and it has been calculated that transpirational cooling accounts for about half of this heat dissipation. (The remainder is lost through convection and conduction.) Hence transpiration is generally regarded as a 'necessary evil'.

1.7 Water conservation: xerophytes and xeromorphic characters

Plants growing in extremely dry habitats ('xeric habitats') are termed xerophytes. They exhibit a number of structural features which are regarded as potentially conserving water and are called xeromorphic characters. Some of these features may also be found in plants of habitats which are not particularly deficient in water. It is common to find xeromorphic characters in evergreen plants of temperate and cold climates, where the plants may suffer water shortage during cold seasons owing to an inability to absorb water adequately by chilled roots, or even because of the soil water freezing. The character of succulence is typical of plants of saline habitats, where water is abundant, but the plants must absorb it from a medium of low C. Xeromorphic characters include:
(1) Deep and/or extensive root systems. These enable the plant to reach water at considerable depths. In the Mediterranean region, tree roots grow right into the porous limestone rock. In some species, the ratio of root weight to shoot weight is very high, as is root length to shoot height.
(2) Water storage organs and tissues: succulence. Any part of the plant may store water – root, stem or leaf. The storage organs are succulent; they contain large, highly vacuolate cells which swell up when water is available and gradually release water to growing regions when there is no external supply, shrinking in the process. Examples of stem succulents are cacti, whilst *Aloe* species and stone crops (*Sedum* spp.) have succulent leaves.
(3) Low surface : volume ratio of shoot organs. Leaves usually are the organs with the highest surface : volume ratios. Xeromorphic plants tend to have small leaves, or succulent leaves, succulence leading to a decrease in the ratio and hence a low water loss by transpiration. Sometimes the leaves lose photosynthetic activity altogether and are replaced by scales or spines – the cacti are the classic example of the latter. The stem then becomes the photosynthetic organ. The possession of spines discourages animals from using the plants.
(4) Hidden stomata. The stomata may lie in deep depressions or grooves, which trap a volume of still air and increase the boundary layer resistance. In dry conditions, the leaves may roll up and protect the stomata. Sometimes the surface cuticle and wax form a dome over the stoma, with a small aperture, again enclosing a region of still air.

(5) Thick cuticle and wax. These clearly serve to conserve water by preventing evaporation through the outer epidermal surface.
(6) Hairiness. The hairs trap air and effectively increase the boundary layer. The hairs also help to keep the temperature down since they are usually colourless or weakly pigmented, resulting in a pale surface which reflects light and decreases heat absorption.
(7) Lignified leaves. When the tissues do become dehydrated, lignification prevents collapse.Several of these features can be present simultaneously. It may be noted that the xeromorphic features are incompatible with fast growth rates. A high root-to-shoot ratio puts a burden on the limited amount of photosynthetic tissues. A low surface : volume ratio as in cacti (and other succulents) results in slow diffusion of CO_2 into the photosynthetic organs and only the outermost cell layers are well illuminated, so that photosynthesis is confined to these layers. In succulent leaves and stems, the ratio of photosynthetic cells to total mass is low. The net assimilation rate is consequently low and growth is slow; cacti are notorious for their low growth rates. The sinking or overarching of stomata, and hairy surfaces, increasing the boundary layer, slow down the inward diffusion of CO_2 even as these features protect against excessive outward diffusion of water. That is the price the plants pay for survival in xeric habitats.

2. Mineral nutrition

2.1 Introduction

Of the naturally occurring 92 elements of the periodic table, about a quarter are essential to plants. Water and CO_2 provide the plant with the elements C, H and O; the remaining necessary elements are obtained by flowering plants as inorganic mineral ions, mostly from the soil solution. Water uptake and ion uptake are to some extent linked, e.g. water uptake mediated by root pressure depends on ion uptake, and the rate of ion uptake tends to increase with increasing rate of transpiration. But the uptake of mineral ions differs greatly from water uptake in that it proceeds against the free energy gradient of the ions and is dependent on metabolic energy. The transport of ions through cellular membranes is mediated by numerous membrane-bound transport proteins which enable the plant to exert considerable control and selectivity over the process. This is vital if the nutritional needs of the plant are to be satisfied. Heterotrophic organisms obtain nearly all their essential elements via plants and the element composition of plants is accordingly of major interest and importance also for human nutrition.

2.2 Essential elements

2.2.1 Definition: macronutrients and micronutrients

An element is classed as essential to a plant if the plant cannot complete its life cycle without it and no other element can substitute for it. The effect of the element must also be direct, i.e. it should not act by promoting the uptake of another essential element, or by retarding the absorption of a toxic one. To test for the essentiality of an element, the test plants must be placed in an environment totally free from that element. In practice this means growing the plants in a liquid culture medium of precisely known composition. Some elements are required in such minute amounts that they are very difficult to eliminate from solutions to levels below those required by plants. Even distilled water, glass of containers, gaseous pollution of the atmosphere and dust particles may provide enough of certain elements to support plant life. Specially purified water, spectroscopically pure chemicals and a filtered air supply must be used. As techniques have been refined, elements have been added gradually to the list of those needed. Whilst the macronutrient elements (see below) were known by the second half of the nineteenth century, the essentiality of chlorine was not established until 1954 . Nickel was added to the essential list even later, in 1987. There is the further problem of the minerals already present in the propagule used to start the culture; the supply in this may suffice for a considerable period of growth. More elements may possibly be added to the current list, which stands at 18–21 (Table 1). The precise number depends on the species studied, some elements being apparently essential for certain species but not for others; it also depends on how strictly the criteria for essentiality are applied. It may well be that some elements, hitherto known to be required by only a few plants, may eventually be found to be universally essential. As shown in Table 1, the essential elements are classified as macronutrients and micronutrients. The macronutrients are required in large amounts relative to the micronutrients; in culture solutions, macronutrients are supplied at 10^{-3} to 10^{-2} mol L^{-1} , whilst the micronutrient concentrations may be as low as 10^{-7} mol L^{-1}. Most of the micronutrients become toxic at quite moderate concentrations, say above 10^{-4} mol L^{-1} .

Table 1.

Element	Symbol	Form absorbed
Essential macronutrients		
Carbon	C	CO_2, CO_3^{2-} HCO_3^- (bicarbonate)
Hydrogen	H	H_2O
Oxygen	O	O_2, H_2O, CO_2
Nitrogen	N	NO_3^- (nitrate), NH_4^+ (ammonium)
Sulphur	S	SO_4^{2-} (sulphate)
Phosphorus	P	$H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-} (phosphates)
Calcium	Ca	Ca^{2+}
Potassium	K	K^+
*Silicon	Si	H_4SiO_4 (silicic acid)
Essential micronutrients		
Iron	Fe	Fe^{2+} (ferrous)
Magnesium	Mg	Mg^{2+}
Manganese	Mn	Mn^{2+}
Copper	Cu	Cu^{2+} (cupric)
Zinc	Zn	Zn^{2+}
Boron	B	H_3BO_3 (boric acid)
Nickel	Ni	Ni^{2+}
*Cobalt	Co	Co^{2+}
*Molybdenum	Mo	MoO_4^{2-} (molybdate)
*Chlorine	Cl	Cl^- (chloride)
*Sodium	Na	Na^+
Beneficial elements		
Selenium	Se	SeO_4^{2-} (selenate)
Rubidium	Rb	Rb^+
Strontium	Sr	Sr^{2+}
Aluminium	Al	Al^{3+}

In addition to essential elements there are beneficial elements, as indicated in Table 1, which are not absolutely

necessary for survival but promote the growth and vigour of plants. Non-essential elements are also taken up by plants; any element present in the environment will be absorbed at least in small amounts. For plants grown in the soil, large amounts of Al and Na are frequently present as these are common in soils. Though inessential, such elements are far from being inert. They influence the ionic balance and osmotic potential of the cells and may affect the uptake of essential ions. Many non-essential elements are toxic in quite low amounts and their uptake is detrimental to the plants and to the animals which feed on them.

2.2.2 The physiological functions of the elements in plants

The roles of the essential elements in plants are at least partly known; the majority of the essential elements are indeed universal for all living organisms and many of their functions are the same in plants, animals, fungi and prokaryotes. In discussing the functions of the elements, most emphasis is here put on those functions that are characteristic of flowering plants. The macronutrient elements are constituents of cellular macromolecules, including all the major building blocks of protoplasm, whereas many micronutrients are enzyme cofactors or occur as parts of prosthetic groups of enzymes. Carbon and hydrogen are of course constituents of all organic molecules and the majority of organic molecules of living cells contain oxygen as well; these three elements are present in the greatest amounts.

Nitrogen, too, is a constituent of many cellular molecules, in particular proteins and nucleic acids, the key macromolecules of life as we know it. There are many lower molecular weight nitrogenous organic compounds vital to cell metabolism – vitamins, cofactors, hormones, the chlorophyll pigments and the phytochrome photoreceptors. Flowering plants additionally contain an extraordinary variety of nitrogenous secondary compounds not involved in basic metabolism. These include alkaloids, among which are compounds used as drugs, e.g. morphine, nicotine and quinine. Plants also contain numerous non-protein amino acids, which are not incorporated into normal proteins. There has been much dispute about the possible physiological functions of the secondary chemicals. Both the alkaloids and the non-protein amino acids are toxic and often bitter tasting; one possible function is protection against herbivores. In seeds, non-protein amino acids, with a high proportion of N by weight, can act as N storage compounds. Some non-photosynthetic pigments contain N, e.g. betacyanin, the red pigment of beetroot (*Beta vulgaris*).

Sulphur performs an important structural role in proteins where the disulphide bridges –S–S– stabilize tertiary protein structures. Sulphydryl groups, –SH, are found in the active sites of many enzymes. There are also –SH-containing coenzymes, e.g. coenzyme A, whilst glutathione, again with a –SH group, is an important antioxidant. Several iron–sulphur proteins, e.g. ferredoxins, occur in the electron transfer systems of chloroplasts and mitochondria; these proteins contain clusters of linked S and Fe atoms at their reactive sites. Membrane sulpholipids are structural molecules which contain a sulphate group, found in chloroplast thylakoid membranes. Numerous flowering plants contain pungent secondary S-containing compounds appreciated as flavours; these are very common in the Brassicaceae (cabbage family) which includes mustard (*Sinapis alba*). Onions (*Allium cepa*), garlic (*Allium sativum*) and related species are also flavoured with S-containing chemicals. The presence of such compounds may deter some herbivores.

Phosphorus is contained in nucleic acids and also in membrane phospholipids which make up the bimolecular lipid leaflet of biological membranes. As a component of the adenosine phosphates (ATP, ADP and AMP) and related nucleotides, the phosphate group is involved in 'energy metabolism' and intermediary metabolism involves many phosphorylated intermediates. In metabolically active cells there is a continuous turnover of phosphate from organic combination to Pi (inorganic phosphate) and back again.

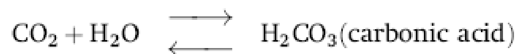
Calcium, as in cells of other kingdoms, contributes to membrane stability in plant cells by its association with membrane phospholipids, and it is necessary for the maintenance of the normal permeability of the plasmalemma. In plants it also contributes to cell wall structure as calcium pectate; this is a major component of the middle lamella which cements adjacent cell walls together. The Ca^{2+} ion is extremely important in stimulus perception; one of the first effects in the chain of reactions set off by a stimulus, environmental or hormonal, is very often a change in the cellular concentration of Ca^{2+} which is termed a 'second messenger'. Ca^{2+} further acts as activator to some enzymes – amylases, ATPases and phospholipases.

Potassium is something of a mystery element. It is present in cells as the free K^+ ion; it does not enter into organic combination. It is known to be the activator of some enzymes, but other elements which act as enzyme activators are required only in micronutrient amounts. The affinity of proteins for K is, however, low and it may be that fairly high concentrations are needed to make enzymepotassium complexes. It is the chief cation of protoplasm and as such it balances the charges on cytoplasmic anions, organic cations being few. Chloroplasts have a high content of K^+; the movement of H^+ from the stroma into the thylakoid lumen during photosynthesis is electrically balanced by a movement of K^+ into the stroma from the cytosol and a shortage of the element leads to a low rate of photosynthesis. The K^+ ion is very important in controlling C (the water potential) and hence the water content of plant cells. Cell expansion is associated with the accumulation of K^+ in vacuoles, which induces water uptake into the vacuoles and an increase in size. In plant cells which function in movements involving turgor changes, K^+ ions are concerned in turgor control; such cells are stomatal guard cells and the pulvinar cells (hinge cells) of leaves and petioles. In these cells, increases and decreases of turgor are achieved by K^+ moving in or out, water following according to the resulting C gradient. Since so many of the effects of K^+ are physical effects on C or electric potential, the question is why K^+ should be so specifically required and why the very similar Na^+ can replace it to only a limited extent.

Silicon in the form of silica gel, a hydrated oxide of Si, gives the cell walls of grasses, including cereals, their characteristic rigidity; this is very conspicuous in the dried-out straw. Si is not known to take part in any biochemical reactions within cells and Si-requiring plants can be nursed to maturity in culture in the absence of the element. Lack of Si does, however, result in some wilting, withering and necrosis, and under natural conditions such Si-deficient plants would have little chance of survival; hence it is reasonable to consider Si as an essential element for species which normally have highly silicified cell walls, e.g. wetland grasses. Many other species contain smaller amounts of Si in their walls and Si can be regarded generally as a beneficial element. It can ameliorate toxic effects of Al and Mn and can increase resistance to fungal disease. Many of the micronutrients have been identified as enzyme activators or as parts of the prosthetic groups of enzymes. Iron and copper are present in the respiratory and photosynthetic electron transfer chain cytochromes. They are also needed for other oxidative enzymes: Fe for catalase and peroxidase, Cu for ascorbic acid oxidase and polyphenol oxidase; Fe is present in iron–sulphur proteins, as mentioned in connection with S and Fe is necessary for chlorophyll synthesis.

Magnesium and manganese activate many dehydrogenases and phosphate transfer enzymes and are also important in photosynthesis, a Mg atom being part of the chlorophyll molecule whereas Mn is present in the O_2-evolving complex. All the three elements Fe, Cu and Mn are transition metals, able to change valency as they lose or gain an electron; hence their association with oxidoreduction activities, which involve the transfer of electrons between reactants.

Zinc is again an activator for many enzymes. Particularly important in plants are alcohol dehydrogenase, superoxide dismutase (which degrades the highly reactive and dangerous superoxide radicals formed during certain oxidative and photosynthetic reactions), and carbonic anhydrase. The last-named catalyses the reaction



$$CO_2 + H_2O \rightleftharpoons H_2CO_3(\text{carbonic acid})$$

In aquatic plants, where carbonate is the main C source for photosynthesis, this reaction produces CO_2 as substrate for Rubisco. In C_4 plants the reverse reaction occurs, resulting in the formation of carbonic acid which dissociates to form bicarbonate, the substrate for PEP carboxylase.
Nickel is a constituent of the enzyme urease, which hydrolyses urea; the enzyme is needed for N metabolism in plants. Molybdenum is present in the enzyme nitrate reductase, which is needed to utilize nitrate, the major source of inorganic N for most plants, and it is needed for symbiotic N_2 fixation. It is also part of the cofactor (Moco) for aldehyde oxidase, an enzyme involved in the synthesis of ABA, and in a few other oxidases. The amount required is extremely small. Cobalt also is needed in minute quantities only, and is known to be needed for symbiotic N_2 fixation, which involves the Cocontaining vitamin B_{12}. Since plants normally associated with symbiotic N_2 fixers can survive without the symbionts, Co might be argued to be beneficial rather than essential. However, symbiotic N_2 fixation is very important not only for the species in which it occurs, but in the overall ecological context. Hence it seems appropriate to include Co in the essential list.

Chlorine is required for the O_2 -evolving system of photosynthesis. For this it is needed in only micronutrient amounts. However, the element is taken up by cells in large quantities and the chloride ion Cl^- is the chief inorganic anion in cells, often accompanying K^+, e.g. during K^+ fluxes in stomatal guard cells, so that it is beneficial in much larger amounts than required to fulfil its essential biochemical role.

Sodium is required for C_4 photosynthesis in some C_4 species where it seems to be involved with the conversion of pyruvate to PEP. It is present in cells as the free Na+ ion and like Cl^- is tolerated in relatively high concentrations. Chemically it is very similar to K and to some extent it can interchange with that element; e.g. in Commelina benghalensis Na can replace K in the control of turgor of the stomatal guard cells. In succulent halophytes, plants which live in saline habitats, Na^+ acts as an osmoregulatory ion, with Cl^-.

Boron is the element for which the physiological role has proved most difficult to elucidate. Much of the B in the plant is associated with cell walls where it cross-links cell wall polymers, such as pectins. It also is needed for normal membrane function. In B-deficient roots, ion uptake capacity deteriorates but when such roots are supplied with B, recovery is considerable by 20 minutes and complete within an hour. Such fast action suggests a primary action at the membrane level, B either affecting membrane permeability or acting on membrane- bound enzymes. There is also some evidence for B affecting enzymes of auxin and ascorbate metabolism. The greatest demand for B is during the reproductive phase; in the absence of B, pollen grain formation fails, and pollen tubes germinating in the absence of B swell and burst.

Regarding the beneficial elements, individual species differ in their requirements. Sodium benefits many species, being able to substitute to some extent for K.

Rubidium and strontium also probably owe their beneficial effect to an ability to replace some of a plant's requirements for K and Ca respectively. Rubidium enhancesgrowth most markedly in K-deficient media. Aluminium is more limited in its beneficial effects; it is beneficial to tea (*Camellia sinensis*), *Fallopia sachalinensis*, and a number of grasses. Selenium is accumulated in large amounts by some 'accumulator species' e.g. certain species of Astragalus growing in Se-rich soils, also by *Lupinus albus* and *Phleum pratense*; it affords some protection against insect attack and protects against toxicity from excess Pi. The beneficial effects of silicon have already been discussed in connection with its essentiality for some species.

Complex interactions take place between mineral elements and metabolism over and above the primary roles of the minerals. Several examples of interactions between mineral supply and growth hormone metabolism have been reported. In sunflower plants, deficiency of N, P or K in the rooting mediumhas been found to decrease the flow of cytokinin hormones from the roots to the

shoots. Macronutrient deficiency can thus act on plant development not only through direct shortage of elements, but via the hormone supply. The biosynthesis of the gaseous hormone ethylene is promoted by Ca^{2+} ions. Ethylene acts antagonistically to the hormone auxin in a number of effects. Thus Ca^{2+} antagonizes auxin by promoting ethylene biosynthesis. Cobalt inhibits the biosynthesis of ethylene and hence Co salts are used to prolong the life of cut flowers, ethylene being normally produced by the flowers and promoting senescence. More such interactions have been reported and undoubtedly more still remain to be noted.

2 .3 Ion uptake and transport in the plant

2 .3 .1 Ions in the soil

With the exception of C, H and O, which are derived from water and CO_2 and are incorporated by photosynthesis, plants acquire all other elements as inorganic ions. Even C can be obtained as the carbonate or bicarbonate ion. Organic nitrogenous compounds can act as the N source, but normally are available to plants in limited amounts if at all. The ions which serve as sources of the essential elements for flowering plants are listed in Table 1. For terrestrial flowering plants the chief source of mineral ions is the soil. The mineral rock particles of the soil yield ions by weathering which gradually brings them into solution; ions are also released by the action of microorganisms on dead organic material. The ion concentration of the soil solution rises as the water content of the soil falls, but except under very dry conditions the solution is very dilute. It has been shown that a solution corresponding in ionic composition to that of a soil solution will support good growth of crop plants provided it is frequently renewed or applied as a flowing solution so that it is not depleted. In a natural soil the ions in the solution are constantly being replenished. The laws of physicochemical equilibrium ensure that, as ions derived from soil solids are removed from the solution, more ions dissolve from the rock particles. Soil Pi concentration is always low, 1 mg L^{-1} or less; it has been calculated that the Pi of the soil solution may need to be renewed 10 or more times per day to meet the P demands of a growing crop. Nitrate also needs rapid replenishment; it is absorbed rapidly by plants, N being needed in larger quantities than most other elements, and being extremely soluble, nitrate is easily washed downwards in rainwater. Not all the ions in the soil are totally free in the soil solution. The colloidal matter in the soil, both inorganic clay particles and organic particles, 'humus', which help to retain water in the soil also serve to retain ions by adsorption. The colloidal constituents of the soil usually carry a net negative charge; cations, being positively charged, are adsorbed to the negatively charged groups on the clay and on the organic particles. These ions are held at the surface of the soil particles by electrostatic attraction only loosely and can be exchanged for other cations; by washing a soil with a concentrated solution of a salt such as NH_4NO_3 the soil cations can be displaced into the solution in exchange for the ammonium ions (NH_4^+). For most of the anions, there is little adsorption because of the lack of positive charges on the soil colloids. There may be some adsorption of phosphate ions, especially of the trivalent PO_4^{3-} which has a high electric charge density, and phosphate ions can also replace hydroxyl and silicate anions in clays.

2.3.2 Ion uptake by the root Adsorption, absorption and accumulation

The region of most active ion uptake by roots is the same as for water uptake, i.e, the young region of the root behind the apical meristem, the root hair region. The uptake of cations begins with the adsorption of the cations to the cell walls where polysaccharides carry negative charges, which attract H^+ ions moving out of cells by the action of proton pumps. These H^+ are then accessible to be exchanged for soil solution cations, which thus become electrostatically bound to the cell walls. The binding sites lie not just on outer surfaces of walls, but inside the capillary spaces within the cell walls; a living cell's wall may be thought of as a hydrated sponge, with much 'wet space' and much adsorptive surface inside it. There is also the possibility of some cation exchange, termed contact exchange, between H^+ ions adsorbed on the root surface and the cations adsorbed onto solid colloidal soil particles. Anions have few adsorption sites in cell walls; they must simply diffuse into the water-filled capillary spaces of the cell walls before passing through the plasmalemma. To some extent, they 'follow' the cations by electrostatic attraction.

The adsorption of ions may then be succeeded by absorption, passage through the plasma membrane. There is an important difference in meaning between these very similar words, adsorption for attraction outside the plasma membrane, absorption for actual entry into the cell, to the inside of the plasma membrane. When the ions are taken up to a greater concentration inside the plasma membrane than outside, this is termed accumulation (Fig. 1).

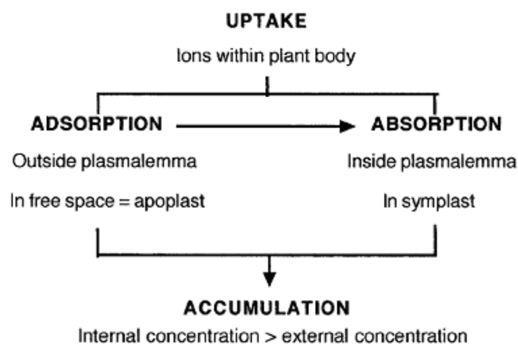


Fig. 1 Processes involved in ion uptake by plant cells

Uptake of ions is by no means confined to root cells. Roots take up the ions in the first instance, but these ions are distributed around the whole plant in the xylem, where they are in the apoplast, outside living cells, and cells in various parts of the plant have to take up ions from the supply carried up in the xylem sap. All living cells of the plant are capable of ion uptake from their environment; in the natural state, for cells other than the outer root cells, the environment for ion uptake is the apoplast.

Compartmentation of the plant cell and the concept of free space

Throughout discussions of ion uptake reference is made to 'inside' and 'outside'. Plant cells are, however, more

complicated than a sac surrounded by a membrane. When plant tissue is thoroughly prewashed and then immersed in a solution containing mineral ions, there is first a very rapid uptake, followed by a much slower rate (Fig. .2). When the immersion takes place at low temperature, around 4^0C, or under anaerobic conditions, only the initial rapid uptake occurs, suggesting that the initial uptake is a physicochemical process not requiring metabolic energy. When the tissue is washed in distilled water after a period of uptake, some of the ions taken up are quickly washed out. These ions are said to occupy the 'water free space' which seems to correspond to the cell walls of the tissue. Some more cations can be removed from the tissue by immersion in a solution of cations, e.g. K^+ might be washed out in exchange for excess NH_4^+. The exchangeable cations are again present in the cell walls, but they are associated with the negative charges on the cell wall polysaccharides and at the plasma membrane surface; these ions are described as being in the 'Donnan free space', named after Donnan, an investigator in this field. A certain fraction of ions is, however, retained firmly; these are the ions which have passed through the plasma membrane. Quantitatively, the fraction that is washed or exchanged out with ease corresponds to the fraction acquired during the rapid uptake phase; the firmly held fraction corresponds to what was taken up in the slow, energy-requiring process. Diffusion and adsorption into the free space or apoplast, being physicochemical processes, do not use metabolic energy and hence proceed even at low temperature or under anaerobic conditions, but passage through the plasma membrane, into the symplast, requires ATP. Only what has passed through the plasma membrane is truly inside the living cell, although free space ions are inside the plant or tissue as a whole.

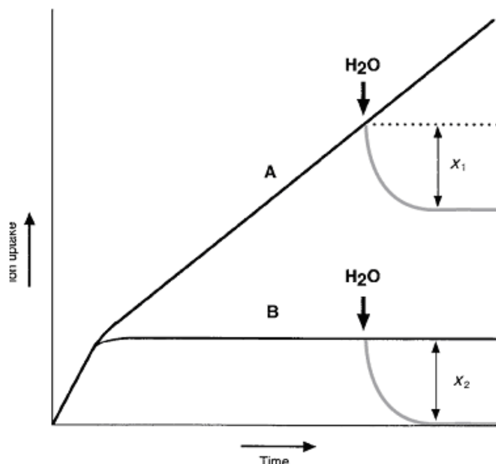


Fig: 2 Diagrammatic representation of a typical time course of ion uptake by plant tissue. Curve (A) under aerobic conditions and at a relatively high temperature, say 15–25^{0}C; curve (B) under anaerobiosis, or in the presence of respiratory inhibitors, or at a low temperature, say 0–5^{0}C. The grey curves indicate what happens when the tissue is placed in distilled water at the time indicated by the arrow. The initial rapid uptake lasts 10–20 minutes and represents uptake into the free space; in water, the free-space ions are washed out and x1 = x2.

The cell wall is not a highly selective barrier to movement of solutes except in so far as, owing to negative charges on the wall polysaccharides, it tends to attract cations. The larger pore spaces between the wall macromolecules reach dimensions up to about 5 nm; the diameters of the ions, including water of hydration, are below 1 nm. The wall does slow down the diffusion of chemicals with masses above some 200–300 Da, but usually transmits molecules up to several thousand Da. The plasma membrane is generally regarded as the primary barrier to ion movement. There are, however, further barriers within plant cells. In mature cells the vacuole forms a compartment occupying 80–90% of the protoplast volume and the tonoplast (vacuolar membrane) is an important barrier to ion movement, for a large proportion of the free inorganic ions in a plant cell passes into the vacuole. The organelle membranes constitute further barriers limiting the free diffusion of ions.

2 .3 .3 The transport of ions within the plant

The long-distance transport of ions takes place in the xylem concurrently with water transport. Just as for water, it has been questioned whether the ions move through the outer root tissues by an apoplastic, symplastic or transcellular route. Apoplastic ion movement could be partly by diffusion, partly along with the flow of water moving into the transpiration stream. The endodermal Casparian strips are believed to form a barrier to ion movement, as for water. Indeed for ions there is more direct evidence for this being so. Solutions of salts of the heavy elements lanthanum (La), lead (Pb) and uranium (U) have been used as tracers for ion movement. These elements can be located by electron microscopy because of their high atomic masses (La = 139, Pb = 207, U = 238) and can be seen in cell walls of the root cortex and in the endodermal cell walls as far as the Casparian strips, but not beyond; La and U are apparently unable to cross the plasmalemma and are excluded from the stele; Pb, however, enters the endodermal cell cytoplasm and also the stele. The above observations strongly support the suppositions that (1) ions move in the apoplast; (2) the Casparian strip is an effective barrier to ion movement; and (3) the endodermis can be crossed by ions only if they can enter the symplast. The weakness of the argument lies in extrapolating from data obtained with these ions of high atomic weight to the behaviour of ions of essential elements, with lower masses. There is, however, indirect evidence for similar behaviour by the nutrient ions. In barley (Hordeum vulgare) roots, Pi ions and K^+, which readily enter the symplast, are translocated to shoots even from the older parts of the roots, where the endodermal Casparian strips are fully developed. The ion Ca^{2+}, which penetrates the symplast with difficulty, is translocated to the shoots mainly from apical regions where the endodermal cell walls are still permeable.

When the cortex was stripped from a segment of barley roots (still attached to the shoots) so as to break the endodermal cells across at the Casparian strips, the concentrations of ^{32}Pi and $^{85}Sr^{2+}$ in the transpiration stream equalized with the external medium, which was not the case when the endodermis was intact. The endodermis can thus regulate the entry of ions into the stele. The endodermis also acts as a barrier against the outward leakage of ions from the stele. Experiments with $^{45}Ca^{2+}$ in barley roots have shown that over 60% of the radioactivity was exchanged out from the cortex for excess unlabelled Ca^{2+}ions in 10 minutes; but from the pericycle

(the cell layer just inside the endodermis), only 19% was lost. In summary, the mineral ions absorbed by roots can travel to the xylem in both the apoplast and the symplast. In the extreme apices of the roots, the apoplastic pathway is continuous, but once the Casparian strips have fully developed (this may occur within a few millimetres of the tip) the endodermis can be crossed only via the symplastic route. In regions of the root where secondary rootlets emerge, the endodermis may be interrupted, giving freer access to the stele again. Control of what passes into the xylem is possible by the selective transfer of ions to the symplast anywhere between the epidermis and the endodermis, by the retention of ions by living root cells, and perhaps by a filtering action in the endodermis. The sap composition is further modified during long-distance transport by the absorption of ions from the flowing stream by living xylem parenchyma cells adjacent to the conducting cells.

When the rate of water transport from the soil to the xylem is increased, particularly by increased transpiration, ion uptake and transport to the shoots are also increased. It may be that the additional ions entering the xylem represent a passive mass flow of ions carried along in the water current through the apoplast. Alternatively, it has been suggested that the dilution of the xylem sap by an increased rate of water uptake into the conducting cells stimulates a higher rate of ion secretion into the sap from the symplast of living xylem cells. A still further possibility is that the tension pull created by a high transpiration rate lowers the resistance to ion (and water) movement through membranes and thereby increases the rate of secretion or of passive leakage from the symplast. There is evidence that tensions developed in the xylem vessels are transmitted across the root diameter.

Movement in the xylem is one-way traffic. In leafy plants, most of the solution in the xylem eventually arrives in the leaves, though some solutes and water are of course absorbed on the way by older root segments, stems and petioles. For the ions moving away from the conducting cells, there are again the two possible pathways: via diffusion through the apoplast, or by absorption into adjacent living cells with subsequent distribution through the symplast.

Some of the elements are retained in the tissues to which they were first transported; these are known as immobile elements. One such example is Ca. Crystals of calcium carbonate or calcium oxalate are often precipitated in vacuoles of older cells of leaves and stems. Silicon is deposited as the insoluble silica and is immobile. In many species B also is not relocated; a common symptom of B deficiency is the death of meristems: once the external supply fails, the newly formed tissues immediately run short, while the older tissues still have the B originally deposited in them. There are, however, species which can translocate and redistribute B in the phloem; in these species the phloem sap contains sugar alcohols, with which B can form complexes. Other ions, the mobile ones, can be transported out of old, senescing tissues and relocated to young, growing regions; K^+ is a mobile ion. This transport occurs in the phloem. Some elements can be moved around the plant in organic combination: N can move as amides or amino acids, S as S-containing amino acids. Proteins in senescing tissues are largely hydrolysed and the soluble amino acids transported to regions of growth and storage, so that most of the N is conserved.

The constant supply of minerals in the xylem stream leads to the accumulation of ions in the leaves. It has been suggested that the shedding of leaves is equivalent to excretion, ridding the plant of waste minerals (and organic waste, too). There occurs also some leaching of minerals by rain. Around trees one can sometimes distinguish a drip-zone flora, which thrives on the leachings from the tree.

2.3 .4 Ion transport across cellular membranes

Active accumulation: the electrochemical potential gradient

Ion uptake usually is a process of ion accumulation, plant cellsacquiring a total ion content greater than that in their environment, and this applies for many individual ions as well. For some ions, the internal concentration can be very much higher than the external; several hundred-fold accumulation is common. Phosphate, which is present in very low concentrations in natural soils, can be accumulated several thousand-fold. This immediately suggests movement against a free energy gradient, for higher concentration of a solute means higher free energy. However, there is another component to the free energy gradient of ions, namely the electric field. For electrically charged particles, an electric potential gradient is a free energy gradient. A cation, being positively charged, will move towards a more electronegative region. An anion, being negatively charged, will move towards an electropositive region. For ions therefore the free energy gradient is the combined electrochemical potential gradient to which both the concentration of the ion (determining its chemical potential) and the electric potential contribute. This is highly relevant to ion uptake by plant cells, because there does exist an electric charge difference across the membranes of cells. As mentioned earlier, positively charged protons are pumped to the outside of plant cells, into the walls; this results in the inside of the plasma membrane being left electronegative with respect to the outside. The potential difference is in the range of 100–250 millivolts (mV). Similarly, pumping of protons from the cytoplasm across the tonoplast into the vacuole makes the cytosol side of the tonoplast electronegative with respect to the vacuolar side.

The cytoplasm is accordingly more electronegative than the apoplast outside it. Therefore, when a cation, say K^+, is found to be in a higher concentration within a cell than outside it, the question arises: has it been moving against the free energy gradient (as suggested by the concentration gradient) or along the free energy gradient (as suggested by the electric potential gradient)? When the overall electrochemical potential gradient is taken into account, it is found that the accumulation of cations, even allowing for the electronegativity of the cytoplasm, is very often, though not exclusively, against the electrochemical potential gradient. For anions, any accumulation into the cytoplasm must be against the free energy gradient, for anions are negatively charged particles moving into a more electronegative area as well as against the concentration gradient. Movement against the free energy gradient is often termed active and requires an input of

energy by the cell. Where an ion is accumulated along the free energy gradient, the cell is still not getting something for nothing, since maintenance of the potential difference across membranes requires energy. As knowledge of the mechanisms of ion uptake has developed, the distinction between active and passive accumulation has become rather blurred. It is perhaps best to regard accumulation generally as an active process, and then to consider, for individual cases, precisely how the energy input is achieved, directly or indirectly.

When concentrations of ions in the cytoplasm and vacuole are analysed separately, and the electric charge difference across the tonoplast is measured, it is found that the movement of ions from the cytoplasm to the vacuole is also an active process. In mature vacuolated plant cells most of the ions of the cell are in fact transferred to the vacuole, which occupies much of the volume inside the cell wall. The vacuolar store is tapped as required. It is also found that ions may be actively transported out of cells. This is commonly the situation with Na^+. Although the cytoplasmic concentration of Na^+ is generally higher than the external, it is usually lower than would be in electrochemical equilibrium with the external medium. Indeed the active transport of ions into the vacuole can also be regarded as being out of the cytoplasm into a non-living space. Some cells are specialized for the outward transport of ions: these include the cells of salt glands of halophytes, which secrete excess Na^+ and Cl^- ions from the plants; and xylem parenchyma and transfer cells, which secrete ions into the xylem conducting cells. Directions of active flux of some nutrient ions across the plasmalemma and the tonoplast of plant cells are shown in Fig. 3 .

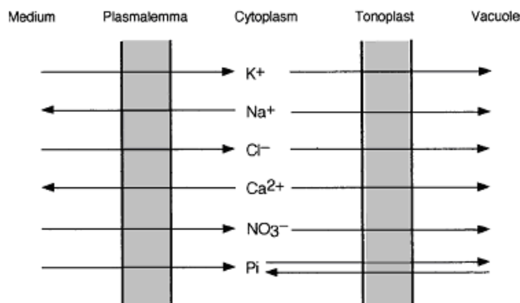


Fig: 3 Directions of active flux of some important ions across the plasmalemma and the tonoplast of plant cells. Phosphate is recorded as Pi since several ionic species may be involved and the (main) species may differ according to the membrane. The direction of movement of Pi between cytosol and vacuole depends on the Pi concentrations in the two compartments: the vacuole acts as a reservoir, releasing Pi to the cytosol when the cytosolic concentration falls low.

The maintenance of physiological concentrations of ions in plant cells is thus an active process requiring an energy supply in the form of ATP. It is estimated that up to half of the energy from root respiration is expended on membrane transport of ions. In photosynthetic tissues, ATP from the photochemical reactions can be utilized to power ion transport.

Mechanisms of membrane transport

The two main characteristics that determine the ease with which a particle can diffuse through a biological membrane are the lipidsolubility of the particle and its molecular size.The more lipid-soluble (lipophilic) and the smaller the particle, the easier the penetration. The mineral ions are very hydrophilic, so they do not dissolve in the lipid bilayer. The atomic weights of some of the nutrient ions are quite low. However, the electric charge on the ions attracts hydration shells of water molecules; e.g. K^+ (mass 39 Da) carries 4 molecules of water whereas the divalent Ca^{2+} (mass 40 Da) has about 12 associated water molecules. These hydration shells increase the effective size of the ion considerably. The permeability of biological membranes towards ions is therefore very low. The flux of ions across membranes is enabled by specific transport proteins in the membranes which facilitate the movement of ions and not only provide the physical means of passage for the ions, but utilize the energy of ATP to transport the ions against their electrochemical potential gradients. The total number of ion transport proteins in plant membranes is much greater than the number of nutrient ions, there often being more than one transport protein for the same ion. In *Arabidopsis*, 16 genes have been identified coding for proteins involved with nitrate uptake, and the same number for phosphate. The specificity of transport proteins varies; some are highly specific to single ions, but often they can transport several related ions, i.e. ions of similar physicochemical properties such as valency and size. The rubidium ion, for instance, is transported by a number of cellular systems for K^+ transport, and the radioactive ^{86}Rb is often used in experiments as a substitute for K^+, there being no convenient K radioisotope available.

The ion transport proteins can be divided into the pumps, the porters (carriers) and the channels; their main characteristics are summarized in Table 2 and Fig. 4 . The term 'carrier' was originally used for all membrane transport proteins, before different types were distinguished.

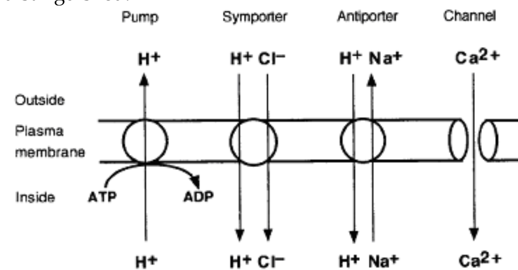


Fig: 4 Ion movement through a membrane via pumps, porters or channels: highly diagrammatic. The plasma membrane is used as the example, but similar systems exist in all cellular membranes. The circles represent the transporters, which are integral transmembrane proteins and may have multiple subunits. Pumps split ATP directly and undergo a conformational change which moves an ion, e.g. H^+, through the membrane. The porter proteins must simultaneously bind a H^+ ion and their specific substrate ion, to move the substrate ion across the membrane, either inwards with the H^+ ion(symport) or outwards in exchange for the H^+ ion(antiport). Some porters require several H^+ per substrate ion.

Table 2 Plant ion transport systems and their basic features. Porters and channels exist also for organic chemicals. All cellular membranes contain transport proteins, mostly specific for a particular membrane.

	Pump	Porter	Channel
Energetics	Uses ATP directly	Uses H^+ gradient from H^+ pump	Movement along free energy gradient
Ions moved s^{-1}	up to 5×10^2	5×10^2 to 10^4	10^6 to 10^8
Examples of ions transported	H^+, Ca^{2+}	Most if not all	Ca^{2+}, K^+, Cl^-, H^+

(1) Pumps. These are transport proteins which hydrolyse ATP and simultaneously transfer an ion across the membrane. The energy is derived from the ATP hydrolysis, directly. Pumps are vectorial, i.e. a particular pump can move an ion only in one direction. The hydrolysis of the ATP results in a conformational change in the pump protein, which causes the transmembrane passage of the ion. Two of the most important ion pumps of plant cells are noted in Table 2 . Ca^{2+} pumps are situated in both the plasma membrane and the tonoplast and they pump Ca^{2+} ions out of the cytosol (Fig. 3), keeping the cytosolic concentration very low, 1-5 $X10^{-7}$ mol L^{-1}, much lower than in the vacuole and the apoplast. Another supremely important ion pump is the proton pump, also known as the proton ATPase. One might not tend to think of H^+ as an important metabolite. In fact the proton pumps constitute the metabolic machinery driving the porters and, in some instances, controlling the channels. Most of the energy utilized in membrane transport in plant cells is via the proton pumps. The previously mentioned movement of protons out of cytoplasm across the plasmalemma and the tonoplast, building up the potential differences across these membranes, is achieved by proton pumps. Their activity of course builds up also a proton gradient. In combination the electric gradient and proton concentration gradient make up an electrochemical potential gradient for protons, favouring the inward movement of the protons. This free energy gradient is harnessed by the porters.

(2) **Porters** are mostly transport proteins which couple the transport of an ion with the inward movement of a proton or protons. The transport can be symport (cotransport), the proton and the ion moving in the same direction, or antiport (countertransport), the two moving in opposite directions (Fig. 4). The carrier in the plasma membrane for Cl^-, for instance, is a symporter, transporting one Cl^- in with $2H^+$. The outward pumping of Na^+ is achieved by an antiporter, which exchanges one proton going in for one Na^+ moved out. There is also a H^+/ K^+ symporter for the entry of K^+ ions into the cytoplasm, amongst the numerous transport systems existing for this ion. The energy input occurs during the activity of the proton pumps which build up the proton gradients.

(3) **Channels.** As the name suggests, channels are formed by proteins with several subunits enclosing an aqueous pore. An open channel is an open hole and movement through a channel is along the free energy gradient; it is very much faster than movement mediated by pumps and porters (Table 2); presumably because of that, channel proteins are present in low numbers per cell (except for aquaporins). The channels nevertheless show specificity; some are extremely specific, for one single ion; others will permit passage according to size. Some interaction is believed to occur between the ion and the channel protein as the ion moves through: '*Passage of an ion through a channel may be likened more to a python swallowing its prey than to a ball rolling through a drainpipe*'. Passage through a channel has been termed uniport since only one chemical moves.

A very important feature of ion channels is that they open only transiently, in response to some stimulus. Any one channel is estimated to be open over only a few per cent of the lifetime of a cell. A permanently open ion channel would be fatal, the cell losing control of its ion concentration. Movement through a channel is along a free energy gradient, so active accumulation would become impossible. (Aquaporins are permanently open and active water accumulation does not occur.) The antibiotic gramicidin acts by inserting into the plasma membrane and forming permanently open channels for K^+ which leak out of the cells. Over 20 ion channels are known from plants. The Ca^{2+} pumps keep the cytosolic Ca^{2+} concentration below the apoplastic and vacuolar levels. Numerous stimuli cause a transient opening of Ca^{2+} channels and flooding of Ca^{2+} ions from the apoplast or internal compartments into the cytosol, where a reaction chain is started. The Ca^{2+} pumps then restore the cytosolic concentration to its previous level. K^+ channels are very important in turgor control of stomatal guard cells. The rapid changes in turgor of motor cells also depend on K^+ movements through K^+ channels.

2.3.5 Control of ion uptake by plant and environment interaction

The ion content and the elemental composition of a plant reflect an interaction between the plant and its environment. Plants show great selectivity: ions are not taken up in the proportions in which they are present in the surroundings (Table 3). For example, most flowering plants show a strong preference for K^+ over Na^+ and maintain a higher internal K^+ concentration irrespective of the external proportions of the two ions. There are accumulator species which concentrate some element to a particularly high degree; the selenium accumulators mentioned earlier accumulate Se to 200 times higher levels than non-accumulators in the same habitat. When several species are grown with an identical external ion supply, each shows a different internal content of mineral elements (Table 3). Sometimes a specific preference can be interpreted in terms of function; in a pasture, the grasses contain much higher levels of Si than other herbs, and this is correlated with the presence of silica in the grass cell walls, on which the grasses depend for rigidity. But the physiological significance of, say, maize sap having double the K^+ content of bean sap in the experiment illustrated in Table 3 is unknown.

The rate of uptake of an ion is dependent on the physiological requirements of the plant. The rate of nitrate uptake by a grass has been found to vary with the diurnal growth rhythm of the plants, the highest uptake rates coinciding with the maxima of growth rate. There are reports of nitrate uptake remaining approximately constant over a wide range of external concentrations, and of some tendency to maintain more or less constant internal concentrations of several ions – e.g. K^+, Cl^-, phosphate and nitrate. There is a negative feedback

between the tissue content of an ion and the rate of its uptake: other things being equal, plants with a high content of an ion take it up at lower rates than plants with a low internal concentration. Higher external concentrations do usually promote higher ion uptake rates, and result in higher internal concentrations, but not in direct proportion to the external. In one experiment, as the medium Pi concentration was raised from 0.03 to 3 0 mmol L^{-1}, a thousand-fold increase, the shoot P content rose only four-fold from 0.23 to 0.96% of dry weight. Physiological control of ion uptake is thus well documented, but the selectivity of plants is far from absolute and the environment also exerts a very considerable influence on a plant's elemental composition. It was noted above that representatives of different species in the same environment differ in their ion contents; it is equally true that specimens of the same species in different ionic environments acquire distinctive elemental compositions. Inessential ions and toxic ions are absorbed. Not only do these have direct effects, but their uptake may compete with that of essential elements; e.g. selenate competes with phosphate for transport proteins, and arsenate competes with sulphate. Any elements present in the environment will be found in plant tissues, even the artificially produced transuranium elements such as plutonium.

Table 3 The differences between ion contents of plant tissues and t external medium, and between ion contents of different species. Bean a maize plants were placed into a culture medium of identical initial com ition for four days; the plant ion content was measured on sap pressed f roots. The points to note are (1) the ions have been accumulated in the pl to much higher concentrations than present in the original solution; (2 ions have been accumulated in proportions very different from those in original solution; (3) the two species have very different ionic contents.

Ion	Initial concentration in medium (mM)	Concentration in root press sap (mM)	
		maize	bean
K^+	2.00	160	84
Ca^{2+}	1.00	3	10
Na^+	0.32	0.6	6
Pi[a]	0.25	6	12
NO_3^-	2.00	38	35
SO_4^{2-}	0.67	14	6

[a]Pi = inorganic phosphate, which has several ionic forms

The uptake capacity of a plant adapts to the current environmental concentration of that ion. Plants grown at low concentrations of an ion develop an enhanced capacity for absorbing that ion compared to plants grown with an abundant supply. For any one ion there may be several transport systems available, with different affinities for the ion. At low external concentrations, high-affinity systems are activated, which are efficient under these conditions. For K^+, the high-affinity system predominates at external concentrations below 0.5 mmol L^{-1}. The mobilization of high-affinity transport systems involves transcription of genes for the high affinity transport proteins. These can be numerous; in *Arabidopsis*, 6 genes for high-affinity phosphate transporters have been identified. Other responses also enhance uptake in a nutrientdeficient environment, including increased root: shoot ratios, increases in number and length of root hairs and increased development of mycorrhiza.

2.3 .6 Mycorrhiza

The word mycorrhiza means 'fungus-root'. It is the name given to a symbiotic association between a plant root and a fungus, which in most cases enhances the mineral nutrient supply of the plant, whilst the fungus benefits from a supply of organic C from the plant. Mycorrhizal associations in some species have been known for many years; gradually it has become apparent that, far from being an odd exception, the formation of mycorrhizal associations occurs in most species of flowering plants in the field and it is highly beneficial. Fossil evidence indicates the presence of mycorrhiza-like associations already in the primitive terrestrial plants of the Devonian era.

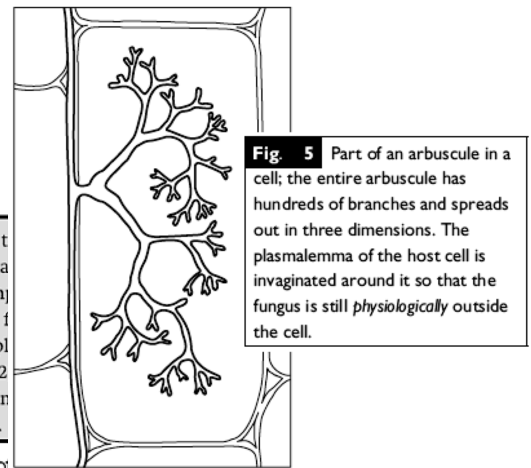


Fig. 5 Part of an arbuscule in a cell; the entire arbuscule has hundreds of branches and spreads out in three dimensions. The plasmalemma of the host cell is invaginated around it so that the fungus is still *physiologically* outside the cell.

In a mycorrhizal association, part of the fungal mycelium is free in the soil, part is closely associated with roots. In ectomycorrhiza there is a thick sheath of fungal mycelium around the outside of young roots, and from this sheath hyphae grow into the intercellular spaces of the root cortex. In endomycorrhiza, all the plant-associated part of the mycelium is inside the root. The most common type of endomycorrhiza is the vesicular–arbuscular (VA) type. The hyphae grow into the cortex cells, penetrating the walls and branching greatly to form the arbuscules ('little trees', Fig. 5), but they do not penetrate the plasma membrane and the cells remain alive. The plasma membrane grows to surround the arbuscules and there is a very large area of surface contact between the plant cell and the fungal arbuscule. Some of the fungal hyphae swell into vesicles, hence the name of vesicular–arbuscular. It is estimated that about 00% of field-grown plants have VA mycorrhiza.

There is also ectendomycorrhiza, with limited cell penetration by the fungus. Special associations occur in some plant groups, e.g. orchids. Exchange of nutrients takes place over the large area of contact between the fungus and the root cells. The surface area of fungus exposed to the soil is also very large, enabling efficient mineral absorption.

Mushrooms (agarics) beneath trees are often the fruiting bodies of ectomycorrhizal fungi. Some fungal species are

associated with particular genera of trees: the brown birch bolete (*Leccinum scabrum*) is confined to birch (*Betula* spp.) whereas others have a broad host range; e.g. the famous red-and-white patterned fly agaric mushroom (Amanita muscaria) is associated with birch, oak (*Quercus* spp.), pine (*Pinus* spp.) and other trees.

Most flowering plants can survive without mycorrhiza, and in laboratory culture with abundant nutrients they grow vigorously, although even in well-nourished cultures the presence of mycorrhiza may enhance growth. But in the field, especially if the soil is deficient in mineral nutrients, growth is very much poorer without mycorrhiza and the plants are less tolerant of stress. The VA association is particularly important in transferring Pi to the plant, whereas ectomycorrhiza is known to provide increased access to both N and P.

The occurrence of mycorrhiza demonstrates how closely organisms in an ecosystem interact. Flowering plants are autotrophic organisms and their basic needs are just light, CO_2 , water and some 20 mineral ions. But as parts of natural communities, the majority of flowering plants are provided with an appreciable proportion of their mineral requirements by mycorrhizal fungi.

3. Translocation of organic compounds

3.1 Introduction

Flowering plants are described as being autotrophic, 'self-feeding', capable of synthesizing all their organic material via photosynthesis. But a flowering plant is a complex organism with cells and organs specialized for diverse functions, and only the green photosynthetic cells are truly autotrophic; they must accordingly supply all the nonphotosynthetic parts with organic carbon. Over small distances, i.e. between individual cells and within small groups of cells, chemicals can move by diffusion through plasmodesmata, or across plasma membranes by diffusion and by active transport. But organic materials must move for long distances; the growing tips of the roots of a tree are many metres away from the nearest photosynthetic leaves and even in a herbaceous plant diffusion would be too slow for the distances involved. We have already seen how water moves in plants over long distances in a specialized transport tissue, the xylem. The subject of this chapter is the long-distance, multidirectional movement or translocation of organic compounds which takes place in the phloem.

3.2 Phloem as the channel for organic translocation

3.2 .1 Evidence for translocation in the phloem

In flowering plants, the xylem is regularly associated with the phloem, the two together making up the vascular tissues. In young organs the two tissues are in contact; when secondary growth occurs they become separated by the vascular cambium, the meristem which then adds xylem to one side and phloem to the other. In woody stems, where the vascular tissues form complete cylinders, it is fairly easy to cut through the outer stem tissues down to the vascular cambium and to remove the 'bark', which includes the phloem, leaving the central xylem intact. Regions of defoliated stem separated from all leaves by such 'bark rings' become deficient in carbohydrates, but the transport of water and minerals continues, showing that xylem transport is still functional. This suggests that the transport of carbohydrates occurs in the phloem. Admittedly the cortex (if present) and periderm are also removed in the bark ring; but these tissues do not contain cells which are structurally suited for long-distance transport. A variation on debarking is to kill the living tissues in a length of woody stem by the application of hot steam. The heat-killed ring stops the translocation of organic substances whilst still permitting xylem transport. This type of experiment shows that organic material is translocated through living cells, as are found in the phloem, in contrast to xylem, which functions with dead conducting cells. The high concentration of organic compounds in phloem sap also strongly supports the postulate of its function in the transport of organic nutrients.

The most direct evidence for phloem as the channel of organic translocation comes from the use of tracers. Various fluorescent dyes, such as fluorescein and its derivatives, can be directly observed under the microscope to move in the phloem. At first this observation was regarded with caution, since these are artificial compounds and might move along paths different from those of natural metabolites. Final confirmation has come from radioactive labelling. When radioactive CO_2 is supplied to photosynthesizing leaves, the radioactivity soon appears in the phloem of the petiole and the stem, as radioactive sugars. Here there has been no introduction of any foreign substance, nor any interference with the plant's normal activity, and the data prove that the products of photosynthesis move from their sites of production in the phloem. Radiolabelling has shown that the phloem is also the pathway of translocation out of non-photosynthetic storage organs. Since it has been established that naturally transported metabolites and numerous fluorescent dyes move along the same pathway, the dyes are now frequently used as tracers for phloem transport.

3.2 .2 The structure of phloem

The phloem of flowering plants consists of several types of cell. Tracer experiments have shown that, at the cellular level, translocation proceeds through the sieve tubes, built of longitudinal files of individual sieve tube elements (sieve tube cells). The sieve tube diameter usually lies between 10 and 50 mmand the length of individual cells is 150–1000 mm, but in palms diameters of 400 mm and lengths of 5000 mm have been reported; in minor veins, however, sieve tubes can be very narrow, below 2 mm. The transverse or oblique end walls between the individual sieve tube cells, some 0.5–2 mm thick, are pierced by pores giving them a sieve-like appearance and are known as the sieve plates, hence the cells' name (Fig. 1). The diameter of the sieve plate pores is extremely variable between species and in different parts of a plant. The narrowest are only 0.1 mm wide; 0.5–1.0 mm might be considered an 'average' value. But in the Cucurbitaceae (marrow family) pore diameters up to 10 mmare found, and 14 mm has been reported for Ailanthus altissima (tree of heaven). Associated with the sieve tube elements there often are companion cells, one or more to each sieve tube cell, lined up longitudinally beside the conducting cells.

The sieve tube elements have a very unusual structure. In the mature state they lack nuclei and the only organelles identifiable are small plastids, sparse mitochondria and endoplasmic reticulum, but these organelles do not occupy much of the cellular volume, lying next to the cell walls. Most of the lumen is filled with a kind of sap but there is no tonoplast and no demarcation of a vacuole. The cells are still, however, bounded by a functional plasmalemma which also lines the sieve plate pores. The sap contains P-proteins (P for phloem), sometimes visible by light microscopy as protein bodies or longitudinal strands, though at least some of the reported strands are now thought to be damage artefacts. The companion cells on the other hand have a full complement of organelles (Fig. 5.1 A, D). They are rich in cytoplasm, with small vacuoles and very numerous, highly cristate mitochondria, which gives them a high potential for metabolic energy production. The sieve tube cell and its adjacent companion cell are derived by longitudinal division of the same mother cell and the pair is often referred to as the sieve element–companion cell (SE–CC) complex. It is generally believed that the companion cell with its nucleus exerts control over the enucleate sieve element. The companion cells are joined to sieve tube cells by abundant plasmodesmata that are unusually large on the sieve element side and branched on the companion cell side (Fig. 1 C). These plasmodesmata also have a very high molecular exclusion limit, i.e. a high limit for the molecular mass that can pass through, much higher than in other plant tissues. The materials that are translocated are found in the companion cells, too; when radioactive photosynthate is being translocated, the companion cells also become radioactive. Other cell types foundin the phloem are phloem parenchyma cells and fibres.

There is some difference in the structure of phloemin various parts of the plant. Photosynthate is loaded into the phloemin the fine minor veins of leaves, and this loading (collecting) phloem has sieve tubes narrower than the companion cells. In the petioles, stems and older parts of roots, the transport phloem sieve tubes are wider than the companion cells. In the unloading (release) phloem, where the solutes leave the transport system, the companion cells are very small and may be absent altogether. There are moreover two basic mechanisms of phloem loading, and phloem structure varies accordingly.

In most perennials with secondary growth, the sieve tubes and their companion cells die after one growing season and are replaced by the cambium in the next season. In perennial monocotyledons, such as the palms, which lack secondary growth, sieve tubes persist many years; in palms 50-year-old sieve tubes have been identified. In a few dicotyledons, too, e.g. lime (*Tilia* sp.) and grapevine (*Vitis vinifera*), the phloem conducting cells survive for several seasons. In such species, the sieve plate pores may be blocked during the winter by a deposit of dormancy callose, a polysaccharide of glucose subunits.

3.2 .3 The composition of phloem sieve tube sap

Obtaining uncontaminated samples of phloem sieve tube sap in quantities adequate for analysis presents problems. Species differ greatly in the ease with which they yield phloem sap. Sometimes exudate is obtained by cutting into the phloem, but this poses the risk of contamination with the contents of other cells. Very often sap fails to exude at all from cut phloem, although exudation may be elicited by mechanical stimulation prior to cutting. The most reliable method of obtaining sieve tube sap from numerous species is to use phloem feeding aphids to tap the sieve tubes. These insects feed by inserting their stylets into sieve tubes from the outside. When the insertion is accomplished, the insect is cut away under anaesthesia. Sap will then continue to drip from the stylets for up to several days, at a rate of 1–2 mL per hour. This liquid has been assumed to represent more-or less unadulterated sieve tube sap. There is evidence that the saliva initially released by the aphid exerts no digestive function on the phloem and the composition of the sap certainly remains unchanged over many hours of collection. There still does remain the possibility of some continuous of liquid from surrounding tissues into the tapped sieve tube units.

Sap analyses vary markedly from species to species. The sap is quite viscous, reflecting its high content of organic solutes; sugars generally make up 90% of the solids and may be present at concentrations of 2 –25% w/v; the water potential is correspondingly low, from –0.6 to –3.4 MPa. In the majority of species, sucrose is the main sugar, often present at 0.4 –0.5 mol L^{-1} , with traces of the oligosaccharides raffinose (a trisaccharide), stachyose or verbascose (two tetrasaccharides); but in some species one of these oligosaccharides predominates. In yet other species, the predominant carbohydrate is a polyol (sugar alcohol) such as sorbitol, dulcitol or mannitol. Glucose on the other hand is found only in very low concentrations and may be undetectable.

Amino acids and amides are regularly present in phloem sap, amounting to 0.2 –12 % of the transported solutes, with glutamate, glutamine, aspartate and asparagine being the most abundant. In the Cucurbitaceae soluble nitrogenous compounds make up a high percentage of the solids, and in perennials nitrogenous compounds are abundant at certain times. Phloem which translocates materials out of seed storage tissues can have high levels of nitrogenous compounds; in Ricinus communis seedlings, a total amino acid/amide content of 0.16 mol L^{-1} has been found, more than half the sucrose content of 0.27 mol L^{-1} . In species where nitrate reduction takes place mainly in the leaves, roots are dependent for their N supply on amino acids and amides translocated down in the phloem. Protein is detectable in the sap in variable amounts and will be discussed separately. Sulphate reduction is located in leaves, roots receiving S-containing amino acids via the phloem. In some plants, different regions of the phloem transport different materials; in cucurbits, phloem within minor leaf veins transportsmainly carbohydrate, whereas phloem strands outside the veins carry mainly amino acids.

Some mineral elements are found in the phloem sap; K^+ is the predominant ion, reaching concentrations of 0.03 –0.5 mol L^{-1} . ATP is a regular constituent. In small quantities, many other compounds have been detected including organic acids, hormones and secondary products; numerous plant viruses, too, spread in the phloem. The pH is usually alkaline at 7.5–8.6, although in perennials it may be faintly acid in the spring. This contrasts with xylem sap and vacuolar saps, which are typically acid.

The carbohydrate concentration of phloem sap derived from photosynthesizing leaves is strongly dependent on the rate of photosynthesis, and hence on weather. It also exhibits regular diurnal variation. In the cotton plant (*Gossypium barbadense*), the highest concentration in the stem is recorded in the latter part of the day. In a number of trees, the highest concentration occurs at night and close to the leaves, a concentration wave moving down the tree.

In perennials there are seasonal patterns in phloem exudation and sap composition. In several tree species, abundant exudate is obtained in late summer but no flow occurs before about mid-June. Marked seasonal changes are found with respect to amino acid content; this is high in spring, drawing on N stored in woody tissues over winter, falls in the summer, and rises to a second peak in the autumn, when leaf proteins break down prior to abscission. The N translocated out of leaves at this time is deposited as organic nitrogenous compounds in the stems, where it remains stored during dormancy. Carbohydrate, too, may be transported to woody stems for storage.

Proteins and RNA in phloem sap

Phloem sap contains proteins, mostly not more than 0.1 mg mL^{-1} , but in the Cucurbitaceae the values reach 10–60 mg mL^{-1} . Gel chromatographic methods have revealed a large number of sap proteins, with molecular masses from 9000 to 2 00 000 Da; over 2 00 protein species have been detected in wheat (*Triticum aestivum*) phloem sap, and individual species exhibit specific patterns. RNA including mRNA can also be detected. Since the sieve tube elements lack a system for transcription or translation, the proteins and RNA must be synthesized in the companion cells and enter through the connecting plasmodesmata. Such movement is vividly demonstrated in transgenic plants programmed to synthesize green fluorescent protein (GFP) in leaf companion cells; the fluorescence appears in the sieve tubes. A very interesting finding is that some proteins in phloem sap have the property of increasing the exclusion limits of plasmodesmata. Proteins from Cucurbita maxima phloem sap were microinjected into individual cells in Cucurbita cotyledon mesophyll, along with fluorescently labelled dextrans, i.e. high molecular mass carbohydrates. These dextrans showed no cell-to-cell movement if injected by themselves. But in the presence of the phloem sap proteins, the spread of fluorescence indicated that dextrans with masses of at least 2 0 000 Da moved through up to 2 0 cells from the site of injection within 1–2 minutes. The accompanying phloem proteins, with masses of up to 100 000 Da, must have moved, too. The exclusion limit for numerous 'ordinary' plant tissues is 800–1000 Da. Phloem proteins from Cucurbita and castor bean (Ricinus communis) also increased the exclusion limits of plasmodesmata in the leaf mesophyll of other species, e.g. *Nicotiana tabacum*. The presence of such proteins in the SE–CC complex presumably maintains the high exclusion limits of the plasmodesmata. Additionally, some proteins in the SE–CC complex may act as chaperonins and unfold large proteins for passage.

Some of the proteins in phloem sap have been identified as enzymes, or as components of the P-protein filaments, and together with others as yet unidentified are presumably necessary for the maintenance of the life functions of the sieve tube cells. The question then arises how such macromolecules avoid being swept with the translocation stream into the sinks: maybe by adsorption on the P-protein fibrils, or to the peripheral ER (endoplasmic reticulum)? But proteins do move in the phloem, even through graft unions. Evidence is mounting that some sieve tube sap proteins and RNA are information molecules destined for transport to the sinks, where they are unloaded. These information molecules include, for instance, signals (believed to be small RNA molecules) which can suppress the activity of specific genes in the sinks. The phloem is emerging as an important carrier of macromolecular information as well as hormonal signals.

3.3 The rate and direction of translocation

3.3 .1 The rate of translocation: velocity and mass transfer

One measure of the rate of translocation is velocity, the distance moved by the translocated material per unit time, expressed in, say, cm h^{-1} . This seems a simple value, but it is difficult to measure. Most estimates to date have been made by introducing a marker into the phloem at a specified point and noting the time of its arrival at another specified point. Fluorescent dyes have been timed, but there is a time delay of unknown length between the external application of the dye and its entry into the sieve tubes, and the dye, as a foreign substance, might conceivably adversely affect the phloem and change the velocity of translocation. The timing of the movement of radioactivity from ^{14}C-labelled photosynthate avoids the introduction of foreign material, but is not straightforward; among other problems, ^{14}C is a weak b-emitter and to detect its presence, plant segments have to be extracted for assay; it is not feasible to hold a detector to the plant. More recently the measurement of nuclear magnetic resonance (NMR) has been utilized; NMR depends on the magnetic properties of atomic nuclei. This technique is used on living plants and causes no damage. The specimen is placed within a detector which gives information about the presence, concentration and movement of selected atoms in known locations in the plant – in this case, the H atoms of the water molecules in the phloem. Actual values of the velocity of translocation, measured in different specimens and using a variety of methods, range from below 10 to 660 cm h^{-1} , with woody plants coming in the lower part of the range, at 20–30 cm h^{-1} . NMR measurements have yielded a value of 200 cm h^{-1} for castor bean (*Ricinus communis*) seedlings and 90 cm h^{-1} for the adult plants.

A somewhat different measure of the rate of translocation is mass transfer, the total mass of translocate moved per unit time. If traffic is used as an analogy, the mass transfer would be equivalent to counting the total number of cars passing an observation point in a given time interval; the velocity is equivalent to the speed at which the cars pass the observation point. The units for mass transfer are g h^{-1} . If the mass transfer is calculated per unit area of phloem, it is termed specific mass transfer (SMT) and expressed in g cm^{-2} h^{-1} . The (specific) mass transfer is measured by increases in dry weight in growing organs. It also is not

easy to measure accurately, for a correction must be applied for the amount of translocate respired away in the organ, and for SMT precise measurements of transporting cell cross-sectional areas are required.

3.3.2 The direction of translocation

In contrast to movement in the xylem, which is a strictly one-way traffic, the direction of translocation in the phloem is variable. Physiologically it is described as passing 'from source to sink', i.e. from organs of synthesis (or organs of storage) to organs of utilization. In a more-or-less mature leafy plant, the mature leaves are sources by virtue of their photosynthetic activity. The main sinks are the growing regions, which are numerous (Fig. 2). The roots and underground perennating organs such as tubers, corms and rhizomes require downward translocation through the shoot axis. In a young vegetative plant, most of the total translocate goes to support the rapidly growing root system. The growing shoot tips, on the other hand, require upward translocation, as do young leaves before they have developed their full photosynthetic capacity. In the reproductive stage, flowers and fruits need upward translocation, fruits in their most rapid growth stage receiving nearly all the translocated nutrients. In the appropriate season, underground storage organs mobilize their reserves and translocate the soluble products upwards to growing shoot organs. Mature axial tissues in stems, petioles and roots of course also need organic nutrients for their maintenance. They draw on the translocation flow as it passes through the axis and are termed axial sinks, to distinguish them from the terminal sinks in the growing regions. Generally, a sink is supplied by the nearest source; thus upper leaves tend to feed the shoot growing points, whilst the roots are fed mainly by the lower leaves. If, however, leaves close to fruits are removed, the fruits start to receive translocate from further away. Tracer experiments have shown that simultaneous upward and downward translocation can occur in the same part of an axis. Since a source = organ of production where solutes are passed into the phloem, and a sink = organ of utilization where solutes are removed from the phloem, it might be postulated that the source-to-sink translocation represents movement along a concentration gradient. There is typically indeed a concentration gradient of translocated material, decreasing from source to sink. However, the situation is more complicated. If a mature leaf is shaded so that it falls below its light compensation point it might be expected to act as a sink, but in fact shaded mature leaves starve, senesce and die. Sugar transport towards a sink is sometimes faster than its utilization in the storage or reproductive organ to which it is moving and temporary accumulation of sugar occurs in petioles and fruit stalks without preventing or reversing the flow. It is obvious that there are metabolic controls for the direction of flow over and above simple concentration gradients. There must also be control of the amount of mass transfer to various sinks.

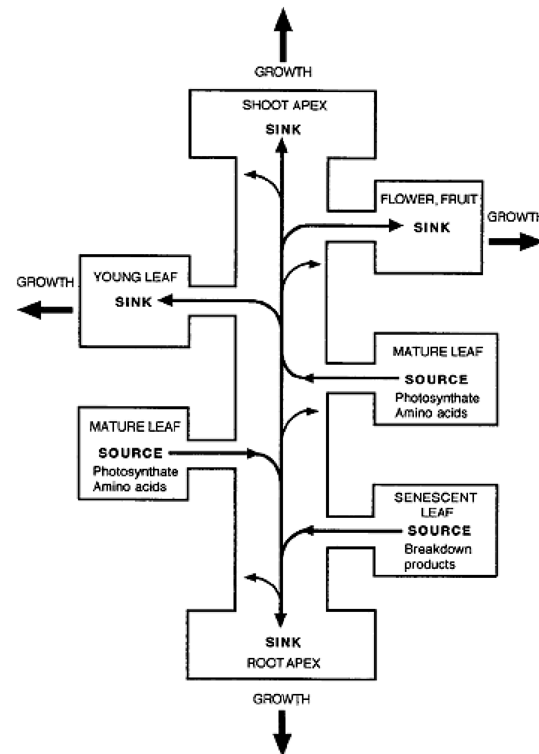


Fig: 2 Phloem translocation 'from source to sink' in a flowering plant. The small curved arrows indicate translocate moving into the axial sinks. Lower mature leaves feed (mainly) the roots, the higher mature leaves feed (mainly) the young leaves and the shoot apex. There are situations when the root, or some other underground storage organ, becomes the main source and the direction of transport is upwards from it.

The overall direction of phloem translocation is under metabolic control; the precise pattern of movement is determined by the arrangement of the vascular tissue. Leaves generally export photosynthetic products to young leaves or floral organs directly above them, and to parts of the root system directly below them. This corresponds to the pattern of vascular connections. Lateral transport from one vascular strand to another hardly ever occurs in either root or stem, and usually the removal of leaves from one side of a plant results in asymmetric, lop-sided growth as a consequence of onesided transport. Lateral diversion is possible in species with a suitably anastomosing vascular system, as occurs in e.g. the beetroot (*Beta vulgaris*).

3.4 Phloem loading and unloading

3.4 .1 Phloem loading

Phloem loading is the transfer of material into the phloem at the source; unloading is the removal of this material from the phloem in the sink. Of the various organs that can act as sources, the minor veins of leaves, which collect the photosynthate from the mesophyll cells, have been studied in most detail. Flowering plant species can be broadly divided into two groups according to their

method of phloem loading in the minor veins: the apoplastic loaders and the symplastic loaders.

In the apoplastic loaders, sucrose is discharged fromthe mesophyll cells into the apoplast (the cell walls), possibly via carriers. Subsequent uptake into the SE–CC complexes is mediated by sucrose-proton symporters in the plasma membranes (Fig. 3); the loading process is thus driven by the proton pumps which pump protons into the apoplast at the expense of ATP hydrolysis. The apoplastic loaders can be anatomically recognized by having very few plasmodesmatal connections between the companion cells and phloem parenchyma or mesophyll cells; their SE–CC complex is a highly isolated unit, relying on membrane transport to move sucrose against a concentration gradient into the phloem. The companion cells of the apoplastic loaders are typically of transfer cell structure, with wall infoldings and hence a large surface (i.e. plasma membrane) area. A number of sucrose transport proteins and their genes has been identified. For example, in rice seedlings (*Oryza sativa*), the sucrose transporter SUT1 is confined to the companion cells, implying that (most of) the sucrose is taken up first by the companion cells and then passes into the sieve tube elements via plasmodesmata. In the potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*), however, SUT1 is distributed throughout the SE–CC complex, so that the sieve elements can take up sugar directly. The expression of the sucrose porter genes occurs when leaves mature and begin to export photosynthate. The repression of sucrose porter gene expression by transforming potato plants with antisense DNA leads to inhibition of sucrose transport, a great accumulation of starch and lipid in the leaves, and severe reduction of root and tuber growth.

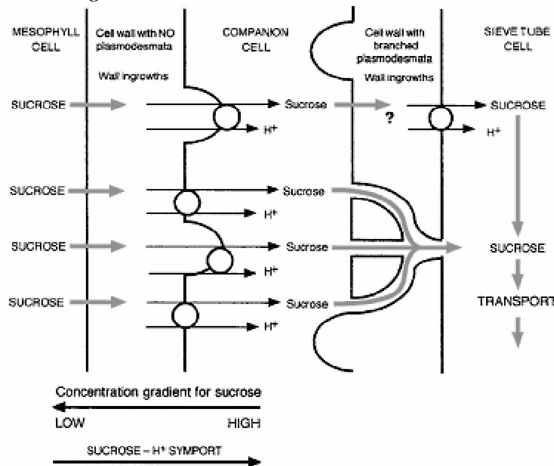


Fig: 3 Apoplastic phloem loading. Sucrose moves (grey arrows) from a mesophyll cell into the cell wall (apoplast) adjoining a companion cell, with wall ingrowths, and is transported by a sucrose-proton symporter (circles) into the companion cell. The sucrose can then diffuse into the sieve tube cell via plasmodesmata, and there are also sucrose-proton symporters in the sieve tube cell plasma membrane which can achieve sucrose transfer. There is an energy input via ATP-splitting proton pumps which pump the protons into the apoplast, and wall ingrowths increase the cell surface area available for transport

The loading of amino acids has been studied less intensely than that of sucrose, but a number of amino acid/H^+ symporters are found in mature leaves.

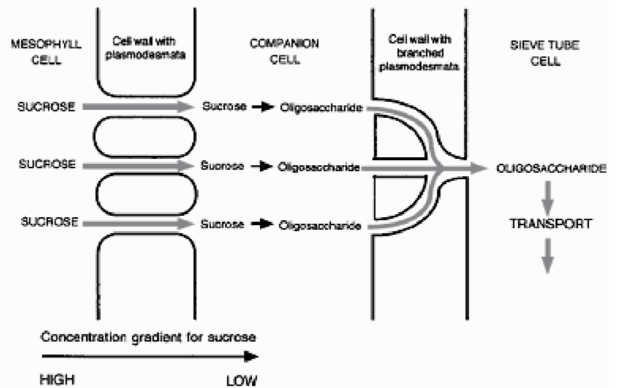


Fig: 4 Symplastic phloem loading by the polymer trap mechanism. Sucrose moves from the mesophyll cell to the companion cell (lacking wall ingrowths) through plasmodesmata by diffusion and possibly mass flow. In the companion cell the sucrose is converted to oligosaccharide, which diffuses through branched plasmodesmata into the sieve tube, but the plasmodesmata on the mesophyll side do not permit back-flow.

They are not specific for single amino acids. In symplastic loaders (Fig. 4) the photosynthate moves to the phloem through plasmodesmata, and there are abundant plasmodesmata between companion cells and phloem parenchyma, and between companion cells and mesophyll cells if in contact; the sieve elements must receive their supply via plasmodesmata from the companion cells since they do not have direct plasmodesmatal connections to other cells. The companion cells lack transfer cell characteristics. The precise mode of movement of the solutes through the plasmodesmata is not certain. It has been noted that symplastic loaders translocate mainly galactose-containing oligosaccharides rather than sucrose, predominantly raffinose and stachyose. Chemically, these are equivalent to sucrose with respectively one and two galactose residues added. It has therefore been suggested that, in symplastic loaders, sucrose diffusing into companion cells is there converted to oligosaccharides, and these are transferred to the sieve element (Fig. 4). This would keep up a concentration gradient for sucrose from mesophyll to the companion cells. This 'polymer trap' hypothesis implies that the oligosaccharides cannot back-flow into the mesophyll through the plasmodesmata, which in the relevant cell walls must have rather low exclusion limits, permitting the passage of sucrose (molecular mass 342 Da) but not of raffinose (504 Da) or stachyose (666 Da).

In some symplastic loaders sucrose is the main carbohydrate translocated. There are not many data on such species, but in two cases at least, willow (*Salix babylonica*) and poplar (*Populus deltoides*) there is evidence that the mesophyll cytosol has a high sucrose concentration and there is a diffusion gradient from mesophyll cytosol to the SE–CC complex. Apoplastic loading is found in many herbaceous plants and in families generally regarded as more highly evolved: Asteraceae (= Compositae), Brassicaceae (= Cruciferae),

Chenopodiaceae, Fabaceae (= Leguminosae) and Solanaceae.Apoplastic loadingis consideredmore efficient. It achieves higher loading rates, is relatively insensitive to low temperatures and is found in temperate-zone species. Symplastic loading is characteristic of the more primitive families, including tropical and subtropical families with many tree species; it is cold-sensitive. Apoplastic loaders, transporting sucrose, can build up higher sugar concentrations in the phloem sap since sucrose is highly soluble; concentrations of 0.5 mol L^{-1} are not rare in phloem sap and values up to 0.9mol L^{-1} have been reported, but raffinose saturates at 0.25 mol L^{-1} .

Osmosis depends on the number ofmolecules per unit volume of solution, so that for the same weight, sucrose, with the smaller molecular mass, gives a lower osmotic potential and therefore a higher turgor pressure, which is postulated to drive translocation in the phloem. Table 5.1 summarizes the features of apoplastic and symplastic loaders.The two types of loading are not mutually fully exclusive, certain extent of apoplastic transfer occurring in symplastic loaders, and vice versa.

Table I Characteristics of plants with predominantly apoplastic or symplastic modes of phloem loading. CC = companion cells.

	Apoplastic	Symplastic
Plasmodesmata between CC and phloem parenchyma or mesophyll	Few	Many
CC of transfer cell type	Yes	No
Main carbohydrates translocated	Sucrose	Oligosaccharides, polyols
Loading rates	Higher	Lower
Loading sensitivity to low temperature	Moderate	Marked
Geographical distribution	Mainly temperate	Many tropical, subtropical
Type of plant	Many herbaceous	Many trees
Taxonomic distribution	Mostly more highly evolved families	Mostly more primitive families

3.4.2 Phloem unloading and post-phloem transport in the sinks

Unloading of translocate from the SE–CC complex can also proceed either symplastically or apoplastically, but in this respect no taxonomic division of species according to unloading type has become apparent; rather, the type of unloading depends on the plant organ, and sometimes also on physiological conditions. In apical meristems and in fruits, unloading is mainly symplastic but with some apoplastic contribution. Symplastic movement has been traced in root apices with fluorescent and radioactive labelling. In unloading phloem the companion cells are small and there is some direct contact between sieve tube elements and other cells. Plasmodesmata pass from sieve tube cells in the root apex protophloem to meristematic cells. In mature stems, unloading from the transport phloem (feeding theaxial tissues) tends to be apoplastic. The movement of sugar out of the SE–CC complex into the apoplast is a simple leakage process, diffusion- driven by the steep concentration gradient. This leakage would in fact deplete the translocation stream, if it were not counteracted by a continuous (partial) retrieval of the sugar by the sucrose-proton symport system. In the terminal sinks, the retrieval capacity of the phloem is low, favouring exit of solutes from the phloem.

After unloading from the transporting cells has taken place, the nutrients must still move from cell to cell, for distances ranging from a few hundred micrometres (e.g. apical meristems) to several centimeters (e.g. some fruits). This post-phloem movement is mainly symplastic. In immature sinks, the exclusion limit of plasmodesmata is very high, up to about 50 000 Da in young leaf tissue (cf. about 1000 Da for mature leaf mesophyll) and 27 000 in developing wheat grains. This enables the distribution of protein and small RNA molecules from the sap through the sink; GFP can be seen to move from the sieve tubes into all tissues of immature leaves. There may be bulk flow of solution through the plasmodesmata, especially in apical meristematic regions, where there is as yet no functional xylem and the phloem, which differentiates much closer to the apex, must supply water as well as nutrients. In root apices, the first sieve tubes differentiate within 250–750 mm of the tip of the meristem, but the first protoxylem matures at 400–8500 mm, or even further away. The possibility of bulk flow in the apoplast, too, has been suggested. The driving force for solute movement would be the diffusion gradient, from the high concentration in the sieve tubes to the sink cells, where the concentration is kept low by utilization in growth and metabolism. Bulk flow could be maintained by the utilization of the water.

3.5 Partitioning of translocate between sinks: integration at the whole-plant level

In addition to control of the direction of translocation, there must be control of how much passes to different sinks at any particular time – i.e. how nutrients are partitioned. Balanced, integrated growth of a plant would otherwise be impossible. The photosynthetic potential of a plant depends on its leaf area; translocation of organic C to the leaf primordia enables leaf growth and increases the photosynthetic potential of the plant. But the growth and subsequent maintenance of the leaves need mineral and water supplies from the roots: during leaf growth there must be translocation of enough photosynthate to the root meristems to support a concomitant increase in the mass (and maintenance cost) of the root system. The organic nutrients available at any particular moment must be partitioned in appropriate proportion between the shoot and root sinks. In fact the growth rates of various parts of a plant can keep in proportion with mathematical precision. This implies that partitioning of organic nutrients, too, is precisely controlled. There must be signalling between the sinks and the sources. Partitioning control is a complex process. Farrar (1996) lists the steps involved in C flow to a sink: 'phloem loading in a source leaf, phloem transport into the sink, unloading and short distance transport within the sink and then metabolism and storage in the sink.' Just about everything that affects the physiology of a plant has the potential to affect partitioning, and control of partitioning is far from being fully understood. One important signalling molecule is sucrose itself (Fig. 5). Sucrose acts in growing regions as a

promoter of the expression of genes involved in growth and respiration (which provides the energy for growth); in the photosynthetic leaves, sucrose represses the expression of genes involved in photosynthesis, including the gene coding for the small subunit of Rubisco. Leaf growth leads to an increased production of sucrose and its translocation to the roots, where the sugar would stimulate the activity of genes which promote growth ofmore root biomass – corresponding to the growth of the leaves. A fall in the sink demand has been shown to decrease the rate of loading at the source,although it is not clear how the messageis transmitted.Turgor has been suggested, a change in turgor at the sink being transmitted to the source and suppressing loading activity.

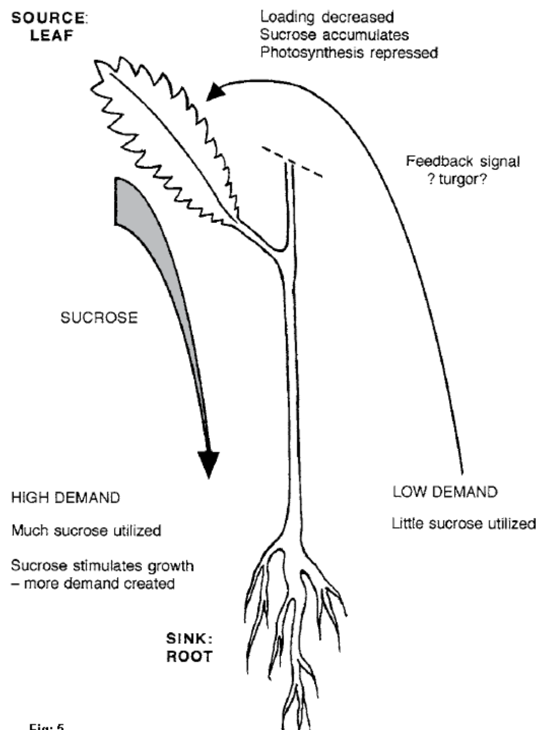


Fig: 5

Sucrose as an information molecule integrating activity between source and sink.

The consequent increase in sugar concentration in the mesophyll would eventually repress the rate of photosynthesis. Some of the proteinsmobile in the phloemmay also act as signals.

The axial sinks can act as buffers between the sources and the terminal sinks: when demand by terminal sinks falls, more photosynthate is unloaded laterally from the transport phloem into the surrounding tissues. The status of various nutrient elements affects the partitioning of photosynthate. When plant growth is limited by a low supply of the elements N, P, S or Fe, the ratio of root to shoot mass rises, more organic C being delivered to the roots, whereas limitation of the supply of Mg, Mn or K leads to a decrease of the root : shoot mass ratio. Water stress increases the root : shoot ratio, root growth being stimulated while shoot growth is reduced.

Hormones are molecules that transmit various signals from one part of the plant to another. Translocates can be attracted to a cut stem stump by applying the hormone auxin to it, or diverted away from a growing wheat ear by the application of auxin to a point remote from the ear. The hormone, however, appears to act by stimulating growth and/or metabolic activity at its point of application, creating a new sink. Cytokinins, too, can induce sink activity. There seems to be no evidence of a hormonal signal specifically and directly controlling loading or unloading.

3.6 The mechanism of phloem translocation

For the movement of any substance in solution, there are two possibilities. Either it is carried along by mass flow (bulk flow) of the solution, or else only the solute moves, e.g. by diffusion, while the solvent remains stationary. The rates of phloem translocation are of the order of 10^5 times faster than diffusion, so this mechanism can be discounted: there must be either mass flow, or some special, rapid transport of the solutes. Movement in the xylem is undisputedly a mass flow of the whole solution, and the motive force is either the tension pull of transpiration, or root pressure. For phloem translocation, too, the current consensus is that mass flow occurs in the sieve tubes, driven by an osmotic gradient. But the structure of sieve tube cells is less obviously suited for mass flow than that of the xylem conducting elements. The hypothesis of mass flow in the phloem needs to be examined critically.

3.6.1 The Munch hypothesis of mass flow driven by an osmotic gradient

One of the earliest theories for the mechanism of phloem translocation was Munch's 1930 hypothesis, which postulated a mass flow along a turgor pressure gradient, driven by a physiologically maintained gradient of osmotic potential (Fig. 6). At the source end, sugars (or other solutes) are loaded into the sieve tubes; this lowers their water potential and induces an inflow of water. The turgor pressure in the sieve tubes rises and the solution is pushed along the phloem by the pressure. At the sink end the solutes are unloaded and water moves out with them, according to the water potential gradient, so that the turgor pressure here is kept low. A lateral movement of solutes along the way into surrounding tissues of stem and root, which all need to be supplied, would also work to maintain a solute concentration gradient (and hence pressure gradient) from source to the final sink. Water would be absorbed into the phloem from the xylem at the source and released at the sink, returning into the xylem, or the water might be utilized at the sink end, as noted earlier.

Evidence in favour of the mass-flow hypothesis

Many observations can be quoted in favour of a mass flow in the phloem. When one single sieve tube unit is pierced by an aphid stylet, the volume of sap exuded in an hour is around 1 mL, equivalent to the volume of some 2500 individual sieve tube cells; the flow can continue for days at this rate. On a macroscopic scale, the phloem sap of sugar palms and agaves is tapped for sugar production or fermentation into an alcoholic drink. One tree can exude

for several months and yield several thousand litres of sap during this period. This is manifestly a mass flow of liquid! Moreover, the sugar concentration remains steady during the period of exudation in both the aphid stylet exudate and in the palm sap, so that the flow of liquid cannot be attributed to a leakage of water into punctured sieve tube cells from the nearby xylem. If mass flow is rejected, all the above-mentioned exudations of sap must be ascribed to an injury reaction unrelated to normal translocation in the phloem. NMR techniques have demonstrated water flow in the phloem in intact plants.

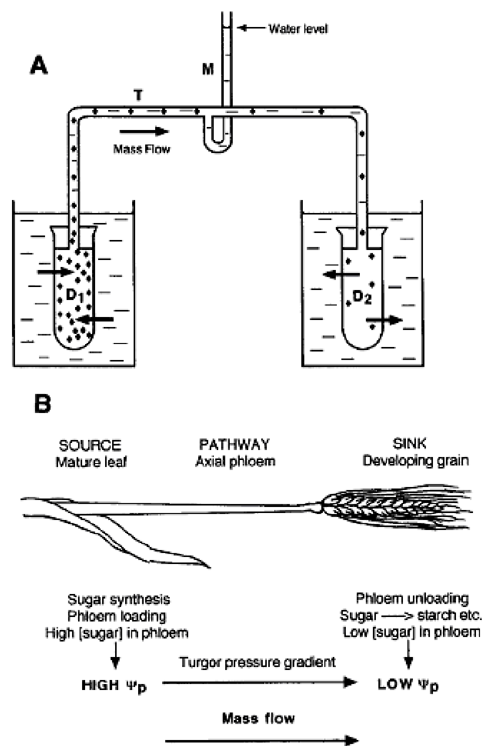


Fig: 5:6 The Mu¨nch mass-flow hypothesis. (A) Principle demonstrated by a simple laboratory experiment. Two dialysis sacs permeable to water but not to sucrose are filled respectively with a concentrated sucrose solution (D1) and a dilute sucrose solution (D2), then connected with glass tubing T and immersed in beakers of distilled water. The greater ψ gradient between water and D1 results in a greater rate of water uptake into this sac; a 'turgor' pressure builds up, solution can be seen to pass along tube T into D2 and pressure is registered by manometer M. The flow stops when the sucrose concentrations in the sacs have equalized. (B) Operation in the plant. A continuous loading of the phloem at the source and unloading at the sink can keep up the turgor gradient and the flow indefinitely.

The Munch hypothesis requires that sieve tube contents should be under a positive turgor pressure, and that there should be a gradient of turgor pressure (and of osmotically active solutes), decreasing from source to sink. The sieve tubes are certainly under positive turgor pressure; this is demonstrated even by the simple fact that sap can exude from cuts. Aphid stylets contain only a very narrow channel, offering a considerable resistance to the flow of the viscous sap; a pressure of 1–3 MPa is required to force the sap through the stylets. Direct measurements of the turgor of sieve tubes have been achieved, either by insertion of sensitive pressure probes, or by the external application of pressure cuffs (analogous to the apparatus used for measuring blood pressure). Such measurements are in practice fraught with great difficulty; nevertheless, not only have positive pressures been recorded, but some workers have succeeded in demonstrating gradients of decreasing turgor passing down trees, away from source leaves. Gradients of sugar concentration have been observed. In soybean (*Glycine max*(soja)) stalks, for example, the sugar concentration in the sieve tubes of the leaflet stalk has been found to be 10.5–12 .5%, when the sieve tubes of the root contained only 4 .4 –6.3 %.

All this is good evidence for the Mu¨ nch mass-flow hypothesis. But the hypothesis cannot be accepted until it has been shown to be feasible quantitatively as well as qualitatively: until it is shown that mass flow at the experimentally measured velocities can be driven by the actually existing pressure gradients, through channels of the dimensions present in the sieve tubes. It is difficult to prove this with complete certainty, the main problem being the interpretation of the fine structure of the sieve plate area.

The dimensions of transport channels in the sieve tube cells Mass flow requires continuous open channels, the wider the better, for the more narrow the diameter, the greater is the frictional resistance to the flow, and the greater is the force that is required to drive the liquid along. The diameter of the sieve tube cells at 10–50 mm would pose no problems. But at each sieve plate the solution must pass through the sieve plate pores. The crucial question for mass flow is: are these pores open channels of sufficient cross-section to permit transport at the observed rates?

In mature sieve tubes the pore diameters, as stated, range from 0.1 to over 10 mm. These are the diameters of the holes in the wall. If it is assumed that the entire hole is open to flow, a hydrostatic pressure gradient of 0.06–0.10 MPam^{-1} has been regarded as adequate to drive mass flow at the measured rates even for narrow pores. (That means, for every metre length along the phloem towards the sink, the pressure in the phloem must decrease by the above value.) Some workers have put the value higher, up to 0.5 MPa m^{-1} . Experimentally measured pressure gradients vary from 0.0_2 to 0.55 MPam^{-1} , which lie within the required range. But if there is any cytoplasmic structure within the pores, much higher pressure gradients become necessary, of tens of MPa m^{-1} . It is clear that turgor-driven mass flow is possible only if the pores are more or less fully unobstructed, at least for species with narrow pores.

Sieve plates and their pores are usually seen lined with a distinctive polysaccharide, callose (a glucose polymer), which may narrow the pore diameters to 0.1 mm even when the aperture in the wall proper is over 1 mm, but it is arguable how much of the callose seen in any preparation was there in the living state, for injury, e.g. resulting from excision, has been shown to induce callose deposition on sieve plates within seconds. Because of this, it can be practically impossible in many instances to know the precise diameter of the pores in a functional sieve plate.

It is even more difficult to decide on the content of a sieve plate pore. The pore diameters are often too close to the limit of resolution of the light microscope for a satisfactory in vivo examination. Sieve tubes cannot be dissected out singly undamaged, and when whole vascular bundles are viewed by conventional light microscopy, clarity is lost. Confocal laser microscopy, however, enables the observer to focus on a narrow plane even in a thick specimen without interference from surrounding material. This method has been used to study living phloem in a leaf still attached to the plant and actively translocating. As far as could be seen the pores were unobstructed, but submicroscopic contents cannot be ruled out. Electron microscopy overcomes the problems of resolution and interference by surrounding structures, but the results remain disputable. The sieve tubes are extremely delicate, being highly hydrated and under high pressure; this makes them very susceptible to fixation damage. In particular, as the pressure is released by cutting, the contents surge towards the cut. According to the method of preparation and the species studied, the appearance of the pores varies. At one extreme pores appear quite empty, or contain some sparse P-protein filaments; at the other extreme, the pores are completely packed with plugs of P-protein and sometimes also with endoplasmic reticulum.

Proponents of the mass-flow theory can regard the empty pores as the natural state and dismiss the densely filled pores as artefacts, containing material swept in as turgor was released on cutting, and/or material coagulated on fixation. Opponents of the mass-flow theory are, however, equally free to argue that empty-looking pores have had their natural contents destroyed by the fixative! Freeze-fracture electron microscopy, where the tissue is frozen extremely rapidly by being plunged into a liquid nitrogen-cooled bath at about –196 ^{0}C, avoids the artefacts caused by chemical fixatives. But in sieve tubes, ice crystal formation has obscured the cell contents and, where structure could be discerned, it has been very variable. In the same sieve plate, some pores may look empty, others are traversed by various amounts of filaments, and some have filaments lying across them. Even 'instantaneous' freezing may not be fast enough to show the true structure for sieve tubes pores through which material is moving rapidly. A translocation velocity of 100 cm h^{-1} is slow on a macroscopic scale; a snail can crawl faster. But relative to cellular dimensions, translocation is very fast. A particle would traverse a 1-mm-thick sieve plate in 0.003 6 seconds (indeed probably less: the measured velocity is an average for the whole sieve tubes and the rate should be even faster in the narrow pores). A fraction of a second would permit structural disturbances to occur in the pores between the start of freezing and final total solidification.

Current view inclines to accept the pores as more or less unoccluded. When damage has been identified with certainty, it has resulted in formation of P-protein strands and deposition of P-protein material on sieve plates rather than loss of discrete structures. This was observed by confocal microscopy when sieve tubes were deliberately wounded. The blocking of pores on wounding confines leakage of sap to the damaged cell.

Metabolic activity in the phloem

According to the original mass-flow hypothesis, the sieve tubes act as passive conducting channels, with no necessity for the expenditure of metabolic energy on the way, for the energy input would take place at the source during loading and possibly also at the sink during unloading. Normally the transport phloem has a high rate of respiration; sieve tube sap contains ATP at an average concentration of 0.4 mmol L^{-1} and the turnover rate of ATP is high, suggesting an expenditure of ATP all along the pathway. This has been quoted as an argument against the mass-flow hypothesis. It has become clear, however, that all along the transport route there is leakage of translocate, and whilst some of this goes to nourish the axial tissues, a major proportion is retrieved by energy-requiring membrane pumps. A high rate of turnover is claimed for phloem proteins and protein synthesis requires an energy supply. Perhaps the unusual state of the sieve tube cells, lacking nuclei, and traversed by a rapid flow of sap, requires a higher than usual rate of maintenance respiration. The high respiratory rate in the phloem therefore cannot be taken as a contraindication to mass flow driven by an osmotic gradient built up locally in the loading region.

The function of the sieve plates

For mass flow, sieve plates appear to be obstructions and their presence has been interpreted as being against the mass-flow hypothesis. They do nevertheless perform an important function in preventing sap loss from damaged phloem: on injury, the deposition of callose narrows the pore diameters, P-protein surges towards the cut because of release of turgor and then piles up against the sieve plates forming slime plugs over the already narrowed pores, making them impassable. Plastids burst, releasing starch grains which help to plug the pores, and possibly they also release chemicals which promote P-protein coagulation.

As noted, from most species there is little exudation from cut phloem in spite of the fact that the sieve tube contents are under considerable hydrostatic pressure. The system can be desensitized by repeated rubbing or even beating in the case of robust woody specimens so that cutting no longer induces blockage, and this effect is utilized when sugar palms are tapped. The sieve plates may also give some mechanical support to the sieve tubes. Different functions for the sieve plates have been suggested in connection with alternative views on the mechanism of phloem translocation.

Alternatives to the mass-flow hypothesis

Alternatives to mass flow have from time to time been proposed. The concept of spreading along interfaces visualizes the translocated molecules forming a monomolecular film on surfaces (P-protein fibrils?), like oil films at an air–water interface. This film would then remain intact, with molecules added at the source end and removed at the sink. The hypothesis does not account for the flow of water, which does occur in the phloem. It is also difficult to conceive of surfaces able to form films with all the variety of compounds that can be carried in the phloem– sugars, amino acids, mineral ions, ATP, fluorescent dyes, hormones, etc. Some sieve tubes

moreover contain very little P-protein. Protoplasmic streaming has been claimed to proceed in the sieve tubes, with transcellular strands running fromcell to cell through the sieve plate pores, but after the original observations were published in the 1960s and 1970s, other workers have been unable to confirm them. The apparent streaming rates of 3 .5 cm h^{-1} are also far too slow compared with the velocity of translocation which reaches several m h^{-1} . Submicroscopic tubules passing through the sieve plate pores, and pumping the tubule contents along by contractions (as in peristalsis) have been proposed without any solid evidence; the same goes for the idea of lashing protein filaments, like miniature cilia, anchored to the outsides of the hypothetical tubules and to sieve tube walls, wafting along a flow in the bulk of the cell. The theory of electroosmosis regards the sieve plates as pumping stations for ions: an electric potential difference (PD) is built up across the plate, by pumping of H+ ions from companion cells into the sieve tube upstream of the plate, and out of the sieve tube into the companion cells downstream of the plate. This would result in the movement of the positively charged K^+ ions in the phloemsap across the plate, towards the negatively charged downstream side and the K^+ ion flow would carry with it water and other solutes. The attractiveness of the electroosmotic hypothesis is that it ascribes both a specific function to the high K^+ content of the phloem sap, and a physiological function to the sieve plates. But the idea has no experimental data to support it. The energy expenditure for the pumping would be high; there would have to be localized differentiation of sieve elements and companion cells with the direction of H^+ ion movement between the cells reversed over a micrometre or two near sieve plates, and all other reported cases of H^+ pumping involve pumping of the ions out of cells. Quantitatively, too, with sieve tube cells often over 100 mm long, it seems hardly credible that so much force could be built up by sieve plates so far apart. In summary, no viable alternative to turgor-driven mass flow in the phloem has been proposed. But it is conceivable that in species with very narrow sieve plate pores there is facilitation of mass flow through the pores from some mechanism like electroosmosis. With sieve pore diameters in the flowering plants covering two orders of magnitude, from 0.1 mm to over 10 mm, the forces required to drive mass flow through the narrowest pores must be very much larger than for the widest ones.

3.6.2 The translocating system in the plant kingdom and macroalgae

All vascular terrestrial plants possess a phloem of elongated living cells joined by some kind of 'sieve areas', i.e. fields of pores on lateral walls, on end walls or on both. In the Psilotaceae, probably the most primitive group of living vascular plants, the sieve cells of the phloem are described as just elongated parenchyma cells with lateral sieve areas. True sieve tube cells, with transverse sieve plates and pores reaching 10-mm dimensions, are found only in the flowering plants. Highly differentiated companion cells are also confined to the flowering plants, although the sieve cells of conifers have associated cells known as albuminous cells. It is reasonable to suppose that the mechanism of organic translocation is essentially the same throughout the kingdom Plantae.

The brown algae (Phaeophyceae) include species with differentiated bodies measurable in metres, and these macroalgae translocate organic compounds through sieve elements, elongate cells joined by pores. In most of the algal species these cells have nuclei and are filled with cytoplasm containing small vacuoles, small plastids and many mitochondria; the sieve plate pore diameters range from 0.03 to 0.1mm. But in the giant kelp (*Macrocystis pyrifera*), which can grow to a length of 50 m, the cells are enucleate, with cytoplasm confined to a peripheral layer, and the pores are much larger, 2 .4 –6.0 mm wide; the pores are lined with callose and callose deposits block themon injury – features strikingly similar to those exhibited by flowering-plant sieve tube cells. The compounds translocated are the algal photosynthetic products – mannitol (and amino acids) in Macrocystis. The direction of translocation is 'source to sink', from mature photosynthetic regions to meristems, and the algal translocation velocities fall within the range measured in flowering plants, 10 cm h^{-1} in Laminaria, 70 cm h^{-1} in Macrocystis. There seems to have been a high degree of parallel evolution of the translocating systems in terrestrial plants and these macroalgae. Both groups share the photosynthetic mode of life, and have a common fundamental structural feature in the possession of a cell wall. When walled cells form a multicellular body, the only possibility of direct intercellular communication is via pores in the cell wall. Once the basic structure of cells joined by communicating pores was established, it is not surprising that these pores should have evolved in several evolutionary lines into a means of large scale transport of nutrients, when transport was necessitated by size increase accompanied by functional differentiation of the body of the green terrestrial plant or alga.

G. Secondary metabolites - Biosynthesis of terpenes, phenols and nitrogenous compounds and their roles.

Plants produce a large, diverse array of organic compounds that appear to have no direct function in growth and development. These substances are known as **secondary metabolites**, *secondary products*, or *natural products*. Secondary metabolites have no generally recognized, direct roles in the processes of photosynthesis, respiration, solute transport, translocation, protein synthesis, nutrient assimilation, differentiation, or the formation of carbohydrates, proteins, and lipids.

Secondary metabolites also differ from primary metabolites (amino acids, nucleotides, sugars, acyl lipids) in having a restricted distribution in the plant kingdom. That is, particular secondary metabolites are often found in only one plant species or related group of species, whereas primary metabolites are found throughout the plant kingdom.

Secondary Metabolites Defend Plants against Herbivores and Pathogens

Many secondary metabolites have been suggested to have important ecological functions in plants:

- They protect plants against being eaten by herbivores (herbivory) and against being infected by microbial pathogens.

- They serve as attractants for pollinators and seeddispersing animals and as agents of plant–plant competition.

Plant Defenses Are a Product of Evolution

- According to evolutionary biologists, plant defenses must have arisen through heritable mutations, natural selection, and evolutionary change. Random mutations in basic metabolic pathways led to the appearance of new compounds that happened to be toxic or deterrent to herbivores and pathogenic microbes.

- As long as these compounds were not unduly toxic to the plants themselves and the metabolic cost of producing them was not excessive, they gave the plants that possessed them greater reproductive fitness than undefended plants had. Thus the defended plants left more descendants than undefended plants, and they passed their defensive traits on to the next generation.

- Interestingly, the very defense compounds that increase the reproductive fitness of plants by warding off fungi, bacteria, and herbivores may also make them undesirable as food for humans. Many important crop plants have been artificially selected for producing relatively low levels of these compounds, which of course can make them more susceptible to insects and disease.

Secondary Metabolites Are Divided into Three Major Groups

Plant secondary metabolites can be divided into three chemically distinct groups: terpenes, phenolics, and nitrogen- containing compounds. Figure 1 shows in simplified form the pathways involved in the biosynthesis of secondary metabolites and their interconnections with primary metabolism.

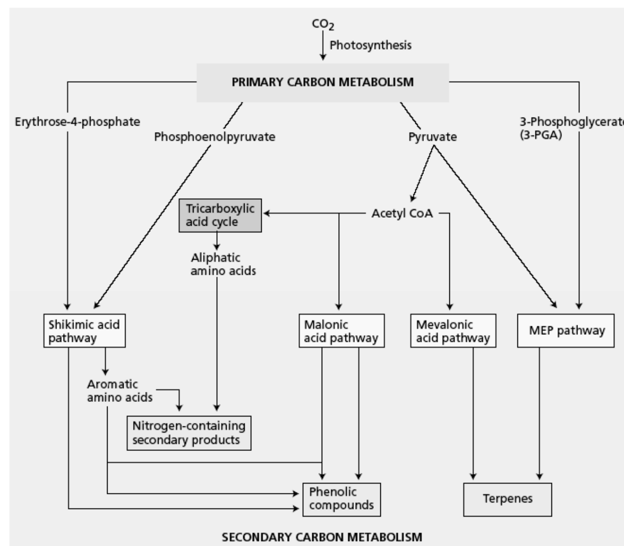


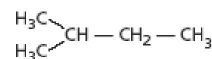
FIGURE 1 A simplified view of the major pathways of secondary-metabolite biosynthesis and their interrelationships with primary metabolism.

I. TERPENES

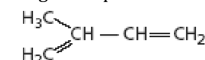
The **terpenes**, or *terpenoids*, constitute the largest class of secondary products. The diverse substances of this class are generally insoluble in water. They are biosynthesized from acetyl-CoA or glycolytic intermediates.

1. Terpenes Are Formed by the Fusion of Five- Carbon Isoprene Units

All terpenes are derived from the union of five-carbon elements that have the branched carbon skeleton of isopentane:



The basic structural elements of terpenes are sometimes called **isoprene units** because terpenes can decompose at high temperatures to give isoprene:



Thus all terpenes are occasionally referred to as *isoprenoids*.

Terpenes are classified by the number of five-carbon units they contain, although extensive metabolic modifications can sometimes make it difficult to pick out the original five-carbon residues. Ten-carbon terpenes, which contain two C_5 units, are called *monoterpenes*; 15-carbon terpenes (three C_5 units) are *sesquiterpenes*; and 20-carbon terpenes (four C_5 units) are *diterpenes.* Larger terpenes include *triterpenes* (30 carbons), *tetraterpenes* (40 carbons), and *polyterpenoids* ($[C_5]_n$ carbons, where $n > 8$).

2 There Are Two Pathways for Terpene Biosynthesis

Terpenes are biosynthesized from primary metabolites in at least two different ways. In the well-studied **mevalonic acid pathway**, three molecules of acetyl-CoA are joined together stepwise to form mevalonic acid (Figure 2. This key six-

carbon intermediate is then pyrophosphorylated, decarboxylated, and dehydrated to yield **isopentenyl diphosphate** (**IPP**2).

IPP is the activated five-carbon building block of terpenes. Recently, it was discovered that IPP also can be formed from intermediates of glycolysis or the photosynthetic carbon reduction cycle via a separate set of reactions called the **methylerythritol phosphate** (**MEP**) **pathway** that operates in chloroplasts and other plastids. Although all the details have not yet been elucidated, *glyceraldehyde-3-phosphate* and two carbon atoms derived from *pyruvate* appear to combine to generate an intermediate that is eventually converted to IPP.

3 Isopentenyl Diphosphate and Its Isomer Combine to Form Larger Terpenes

Isopentenyl diphosphate and its isomer, dimethylallyl diphosphate (DPP), are the activated five-carbon building blocks of terpene biosynthesis that join together to form larger molecules. First IPP and DPP react to give geranyl diphosphate (GPP), the 10-carbon precursor of nearly all the monoterpenes (see Figure 2). GPP can then link to another molecule of IPP to give the 15-carbon compound farnesyl diphosphate (FPP), the precursor of nearly all the sesquiterpenes. Addition of yet another molecule of IPP gives the 20-carbon compound geranylgeranyl diphosphate (GGPP), the precursor of the diterpenes. Finally, FPP and GGPP can dimerize to give the triterpenes (C_{30}) and the tetraterpenes (C_{40}), respectively.

4 Some Terpenes Have Roles in Growth and Development

Certain terpenes have a well-characterized function in plant growth or development and so can be considered primary rather than secondary metabolites.

- For example, the **gibberellins**, an important group of plant hormones, are diterpenes.
- **Sterols** are triterpene derivatives that are essential components of cell membranes, which they stabilize by interacting with phospholipids.
- The red, orange, and yellow **carotenoids** are tetraterpenes that function as accessory pigments in photosynthesis and protect photosynthetic tissues from photooxidation.
- The hormone **abscisic acid** is a C_{15} terpene produced by degradation of a carotenoid precursor.
- Long-chain polyterpene alcohols known as ***dolichols*** function as carriers of sugars in cell wall and glycoprotein synthesis.
- Terpene-derived side chains, such as the **phytol side** chain of chlorophyll, help anchor certain molecules in membranes.

Thus various terpenes have important primary roles in plants. However, the vast majority of the different terpene structures produced by plants are secondary metabolites that are presumed to be involved in defense.

5 Terpenes Defend against Herbivores in Many Plants

Terpenes are toxins and feeding deterrents to many plant feeding insects and mammals; thus they appear to play important defensive roles in the plant kingdom.

- For example, the monoterpene esters called **pyrethroids** that occur in the leaves and flowers of *Chrysanthemum* species show very striking insecticidal activity. Both natural and synthetic pyrethroids are popular ingredients in commercial insecticides because of their low persistence in the environment and their negligible toxicity to mammals.

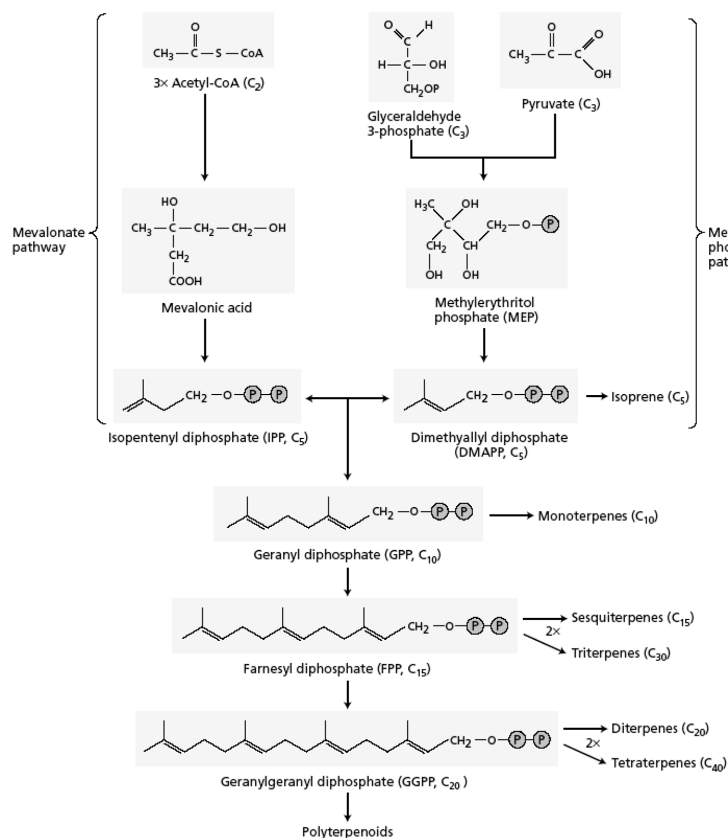


FIGURE 2 Outline of terpene biosynthesis. The basic 5-carbon units of terpenes are synthesized by two different pathways. The phosphorylated intermediates, IPP and DMAPP, are combined to make 10-carbon, 15-carbon and larger terpenes.

- In conifers such as pine and fir, monoterpenes accumulate in resin ducts found in the needles, twigs, and trunk.These compounds are toxic to numerous insects, including bark beetles, which are serious pests of conifer species throughout the world.

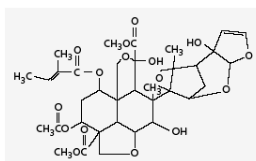
- Many plants contain mixtures of volatile monoterpenes and sesquiterpenes, called **essential oils**, that lend a characteristic odor to their foliage. Peppermint, lemon, basil, and sage are examples of plants that contain essential oils. The chief monoterpene constituent of peppermint oil is menthol; that of lemon oil is limonene.

- Essential oils have well-known insect repellent properties. They are frequently found in glandular hairs that project outward from the epidermis and serve to “advertise” the toxicity of the plant, repelling potential herbivores even before they take a trial bite. In the glandular hairs, the terpenes are stored in a modified extracellular space in the cell wall. Essential oils can be extracted from plants by steam distillation and are important commercially in flavoring foods and making perfumes.

- Recent research has revealed an interesting twist on the role of volatile terpenes in plant protection. In corn, cotton, wild tobacco, and other species, certain monoterpenes and sesquiterpenes are produced and emitted only after insect feeding has already begun. These substances repel ovipositing herbivores and attract natural enemies, including predatory and parasitic insects, that kill plant-feeding insects and so help minimize further damage. Thus, volatile terpenes are not only defenses in their own right, but also provide a way for plants to call for defensive help from other organisms. The ability of plants to attract natural enemies of plant-feeding insects shows promise as a new, ecologically sound means of pest control.

- Among the nonvolatile terpene antiherbivore compounds are the **limonoids**, a group of triterpenes (C_{30}) well known as bitter substances in citrus fruit. Perhaps the most powerful deterrent to insect feeding known is *azadirachtin* (Figure 3A), a complex limonoid from the neem tree (*Azadirachta indica*) of Africa and Asia. Azadirachtin is a feeding deterrent to some insects at doses as low as 50 parts per billion, and it exerts a variety of toxic effects. It has considerable potential as a commercial insect control agent because of its low toxicity to mammals, and several preparations containing azadirachtin are now being marketed in North America and India.

(A) Azadirachtin, a limonoid



(B) α-Ecdysone, an insect molting hormone

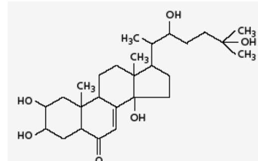


FIGURE 3 Structure of two triterpenes, azadirachtin (A), and α-ecdysone (B), which serve as powerful feeding deterrents to insects.

- The **phytoecdysones**, first isolated from the common fern, *Polypodium vulgare*, are a group of plant steroids that have the same basic structure as insect molting hormones (Figure 3B). Ingestion of phytoecdysones by insects disrupts molting and other developmental processes, often with lethal consequences.

- Triterpenes that are active against vertebrate herbivores include cardenolides and saponins.

- **Cardenolides** are glycosides (compounds containing an attached sugar or sugars) that taste bitter and are extremely toxic to higher animals.

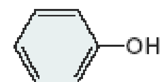
- In humans, they have dramatic effects on the heart muscle through their influence on **Na^+/K^+-activated ATPases**. In carefully regulated doses, they slow and strengthen the heartbeat. Cardenolides extracted from species of foxglove (*Digitalis*) are prescribed to millions of patients for the treatment of heart disease.

- **Saponins** are steroid and triterpene glycosides, so named because of their soaplike properties. The presence of both lipid-soluble (the steroid or triterpene) and water soluble (the sugar) elements in one molecule gives saponins detergent properties, and they form a soapy lather when shaken with water.

- The toxicity of saponins is thought to be a result of their ability to form complexes with sterols. Saponins may interfere with sterol uptake from the digestive system or disrupt cell membranes after being absorbed into the bloodstream.

II. PHENOLIC COMPOUNDS

Plants produce a large variety of secondary products that contain a phenol group—a hydroxyl functional group on an aromatic ring:



These substances are classified as phenolic compounds. Plant **phenolics** are a chemically heterogeneous group of nearly 10,000 individual compounds: Some are soluble only in organic solvents, some are water-soluble carboxylic acids and glycosides, and others are large, insoluble polymers.

In keeping with their chemical diversity, phenolics play a variety of roles in the plant. After giving a brief account of phenolic biosynthesis, we will discuss several principal groups of phenolic compounds and what is known about their roles in the plant. Many serve as defense compounds against herbivores and pathogens. Others function in mechanical support, in attracting pollinators and fruit dispersers, in absorbing harmful ultraviolet radiation, or in reducing the growth of nearby competing plants.

1 Phenylalanine Is an Intermediate in the Biosynthesis of Most Plant Phenolics

Plant phenolics are biosynthesized by several different routes and thus constitute a heterogeneous group from a metabolic point of view. Two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway (Figure 4). The shikimic acid pathway participates in the biosynthesis of most plant phenolics. The malonic acid pathway, although an important source of phenolic secondary products in fungi and bacteria, is of less significance in higher plants.

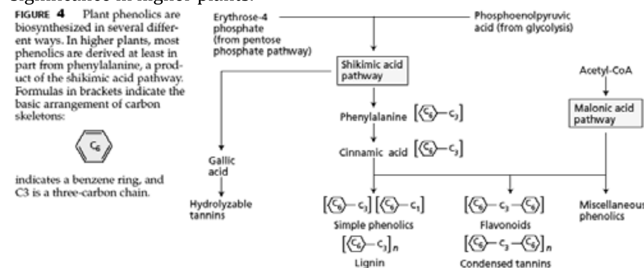


FIGURE 4 Plant phenolics are biosynthesized in several different ways. In higher plants, most phenolics are derived at least in part from phenylalanine, a product of the shikimic acid pathway. Formulas in brackets indicate the basic arrangement of carbon skeletons: C_6 indicates a benzene ring, and C3 is a three-carbon chain.

The **shikimic acid pathway** converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the **aromatic amino acid**. One of the pathway intermediates is shikimic acid, which has given its name to this whole sequence of reactions. The well-known, broadspectrum **herbicide glyphosate** (available commercially as Roundup) kills plants by blocking a step in this pathway. The shikimic acid pathway is present in plants, fungi, and bacteria but is not found in animals.

Animals have no way to synthesize the three aromatic amino acids—phenylalanine, tyrosine, and tryptophan—which are therefore essential nutrients in animal diets.

The most abundant classes of secondary phenolic compounds in plants are derived from phenylalanine via the elimination of an ammonia molecule to form cinnamic acid

(Figure 5). This reaction is catalyzed by **phenylalanine ammonia lyase** (**PAL**), perhaps the most studied enzyme in plant secondary metabolism. PAL is situated at a branch point between primary and secondary metabolism, so the reaction that it catalyzes is an important regulatory step in the formation of many phenolic compounds.

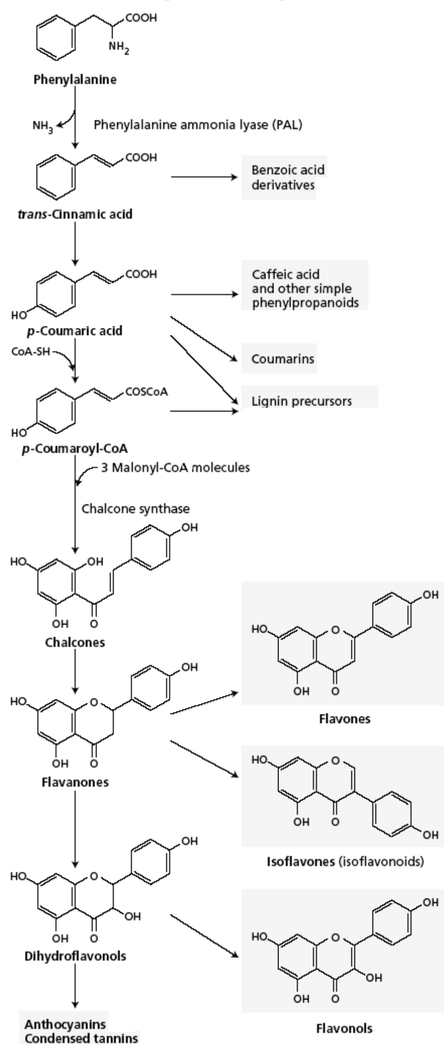


FIGURE 5 Outline of phenolic biosynthesis from phenylalanine. The formation of many plant phenolics, including simple phenylpropanoids, coumarins, benzoic acid derivatives, lignin, anthocyanins, isoflavones, condensed tannins, and other flavonoids, begins with phenylalanine.

The **activity of PAL is increased by environmental factors**, such as low nutrient levels, light (through its effect on phytochrome), and fungal infection. The point of control appears to be the initiation of transcription. Fungal invasion, for example, triggers the transcription of messenger RNA that codes for PAL, thus increasing the amount of PAL in the plant, which then stimulates the synthesis of phenolic compounds.

The regulation of PAL activity in plants is made more complex by the existence in many species of multiple PAL encoding genes, some of which are expressed only in specific tissues or only under certain environmental conditions.

Reactions subsequent to that catalyzed by PAL lead to the addition of more hydroxyl groups and other substituents. *Trans*-cinnamic acid, *p*-coumaric acid, and their derivatives are simple phenolic compounds called **phenylpropanoids** because they contain a benzene ring:

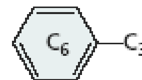
and a three-carbon side chain. Phenylpropanoids are important building blocks of the more complex phenolic compounds.

Now that the biosynthetic pathways leading to most widespread phenolic compounds have been determined, researchers have turned their attention to studying how these pathways are regulated. In some cases, specific enzymes, such as PAL, are important in controlling flux through the pathway. Several transcription factors have been shown to regulate phenolic metabolism by binding to the promoter regions of certain biosynthetic genes and activating transcription. Some of these factors activate the transcription of large groups of genes.

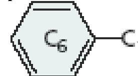
2 Some Simple Phenolics Are Activated by Ultraviolet Light

Simple phenolic compounds are widespread in vascular plants and appear to function in different capacities. Their structures include the following:

• Simple phenylpropanoids, such as *trans*-cinnamic acid, *p*-coumaric acid, and their derivatives, such as caffeic acid, which have a basic phenylpropanoid carbon skeleton (Figure 6A).



• Phenylpropanoid lactones (cyclic esters) called *coumarins*, also with a phenylpropanoid skeleton (Figure 6B).



• Benzoic acid derivatives, which have a skeleton: which is formed from phenylpropanoids by cleavage of a two-carbon fragment from the side chain (Figure 6 C).

As with many other secondary products, plants can elaborate on the basic carbon skeleton of simple phenolic compounds to make more complex products.

- Many simple phenolic compounds have important roles in plants as defenses against insect herbivores and fungi. Of special interest is the phototoxicity of certain coumarins called **furanocoumarins**, which have an attached furan ring.
- These compounds are not toxic until they are activated by light. Sunlight in the ultraviolet A (UV-A) region (320–400 nm) causes some furanocoumarins to become activated to a high-energy electron state. Activated furanocoumarins can insert themselves into the double helix of DNA and bind to the pyrimidine bases cytosine and thymine, thus blocking transcription and repair and leading eventually to cell death.
- Phototoxic furanocoumarins are especially **abundant in members of the Umbelliferae family**, including celery, parsnip, and parsley. In celery, the level of these compounds can increase about 100-fold if the plant is

stressed or diseased. Celery pickers, and even some grocery shoppers, have been known to develop skin rashes from handling stressed or diseased celery. Some insects have adapted to survive on plants that contain furanocoumarins and other phototoxic compounds by living in silken webs or rolled-up leaves, which screen out the activating wavelengths.

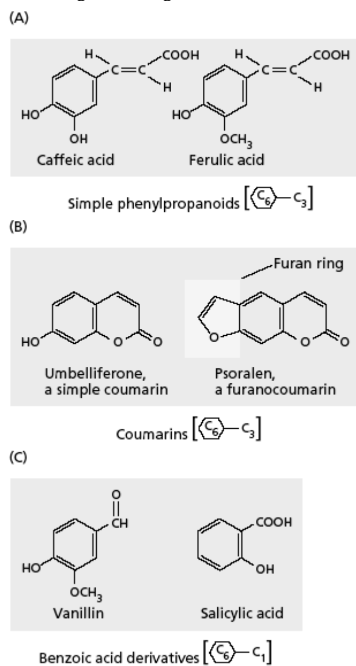


FIGURE 6 Simple phenolic compounds play a great diversity of roles in plants. (A) Caffeic acid and ferulic acid may be released into the soil and inhibit the growth of neighboring plants. (B) Psoralen is a furanocoumarin that exhibits phototoxicity to insect herbivores. (C) Salicylic acid is a plant growth regulator that is involved in systemic resistance to plant pathogens.

3 The Release of Phenolics into the Soil May Limit the Growth of Other Plants

From leaves, roots, and decaying litter, plants release a variety of primary and secondary metabolites into the environment. Investigation of the effects of these compounds on neighboring plants is the study of **allelopathy**.

If a plant can reduce the growth of nearby plants by releasing chemicals into the soil, it may increase its access to light, water, and nutrients and thus its evolutionary fitness. Generally speaking, the term *allelopathy* has come to be applied to the harmful effects of plants on their neighbors, although a precise definition also includes beneficial effects.

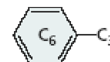
Simple phenylpropanoids and benzoic acid derivatives are frequently cited as having allelopathic activity. Compounds such as caffeic acid and ferulic acid occur in soil in appreciable amounts and have been shown in laboratory experiments to inhibit the germination and growth of many plants.

In spite of results such as these, the importance of allelopathy in natural ecosystems is still controversial. Many scientists doubt that allelopathy is a significant factor in plant–plant interactions because good evidence for this phenomenon has been hard to obtain. It is easy to show that extracts or purified compounds from one plant can inhibit the growth of other plants in laboratory experiments, but it has been very difficult to demonstrate that these compounds are present in the soil in sufficient concentration to inhibit growth. Furthermore, organic substances in the soil are often bound to soil particles and may be rapidly degraded by microbes.

In spite of the lack of supporting evidence, allelopathy is currently of great interest because of its potential agricultural applications. Reductions in crop yields caused by weeds or residues from the previous crop may in some cases be a result of allelopathy. An exciting future prospect is the development of crop plants genetically engineered to be allelopathic to weeds.

4 Lignin Is a Highly Complex Phenolic Macromolecule

After cellulose, the most abundant organic substance in plants is **lignin**, a highly branched polymer of phenylpropanoid groups



that plays both primary and secondary roles. The precise structure of lignin is not known because it is difficult to extract lignin from plants, where it is covalently bound to cellulose and other polysaccharides of the cell wall.

Lignin is generally formed from three different phenylpropanoid alcohols: coniferyl, coumaryl, and sinapyl, alcohols which are synthesized from phenylalanine via various cinnamic acid derivatives.

The phenylpropanoid alcohols are joined into a polymer through the action of enzymes that generate free-radical intermediates.

The proportions of the three monomeric units in lignin vary among species, plant organs, and even layers of a single cell wall. In the polymer, there are often multiple C—C and C—O—C bonds in each phenylpropanoid alcohol unit, resulting in a complex structure that branches in three dimensions.

Unlike polymers such as starch, rubber, or cellulose, the units of lignin do not appear to be linked in a simple, repeating way. However, recent research suggests that a guiding protein may bind the individual phenylpropanoid units during lignin biosynthesis, giving rise to a scaffold that then directs the formation of a large, repeating unit .

Lignin is found in the cell walls of various types of supporting and conducting tissue, notably the tracheids and vessel elements of the xylem. It is deposited chiefly in the thickened secondary wall but can also occur in the primary wall and middle lamella in close contact with the celluloses and hemicelluloses already present.

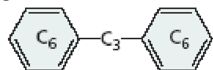
The mechanical rigidity of lignin strengthens stems and vascular tissue, allowing upward growth and permitting water and minerals to be conducted through the xylem under negative pressure without collapse of the tissue.

Because lignin is such a key component of water transport tissue, the ability to make lignin must have been one of the most important adaptations permitting primitive plants to colonize dry land.

Besides providing mechanical support, lignin has significant protective functions in plants. Its physical toughness deters feeding by animals, and its chemical durability makes it relatively indigestible to herbivores. By bonding to cellulose and protein, lignin also reduces the digestibility of these substances. Lignification blocks the growth of pathogens and is a frequent response to infection or wounding.

5 There Are Four Major Groups of Flavonoids

The **flavonoids** are one of the largest classes of plant phenolics. The basic carbon skeleton of a flavonoid contains 15 carbons arranged in two aromatic rings connected by a three-carbon bridge:



This structure results from two separate biosynthetic pathways: the shikimic acid pathway and the malonic acid pathway (Figure 7).

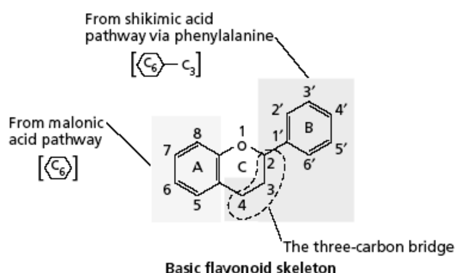


FIGURE 7 Basic flavonoid carbon skeleton. Flavonoids are biosynthesized from products of the shikimic acid and malonic acid pathways. Positions on the flavonoid ring system are numbered as shown.

Flavonoids are classified into different groups, primarily on the basis of the degree of oxidation of the three-carbon bridge. We will discuss four of the groups shown in Figure 5: **the anthocyanins, the flavones, the flavonols, and the isoflavones.**

The basic flavonoid carbon skeleton may have numerous substituents. Hydroxyl groups are usually present at positions 4, 5, and 7, but they may also be found at other positions. Sugars are very common as well; in fact, the majority of flavonoids exist naturally as glycosides.

Whereas both hydroxyl groups and sugars increase the water solubility of flavonoids, other substituents, such as methyl ethers or modified isopentyl units, make flavonoids lipophilic (hydrophobic). Different types of flavonoids perform very different functions in the plant, including pigmentation and defense.

5.1 Anthocyanins Are Colored Flavonoids That Attract Animals

In addition to predator–prey interactions, there are mutualistic associations among plants and animals. In return for the reward of ingesting nectar or fruit pulp, animals perform extremely important services for plants as carriers of pollen and seeds. Secondary metabolites are involved in these plant–animal interactions, helping to attract animals to flowers and fruit by providing visual and olfactory signals.

The colored pigments of plants are of two principal types: carotenoids and flavonoids.

Carotenoids, as we have already seen, are yellow, orange, and red terpenoid compounds that also serve as accessory pigments in photosynthesis.

Flavonoids are phenolic compounds that include a wide range of colored substances.

The most widespread group of pigmented flavonoids is the **anthocyanins**, which are responsible for most of the red, pink, purple, and blue colors observed in plant parts. By coloring flowers and fruits, the anthocyanins are vitally important in attracting animals for pollination and seed dispersal.

Anthocyanins are glycosides that have sugars at position 3 (Figure 8B) and sometimes elsewhere. Without their sugars, anthocyanins are known as **anthocyanidins** (Figure 8A).

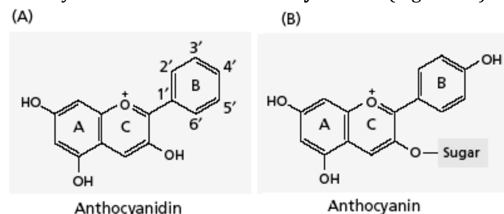


FIGURE 8 The structures of anthocyanidins (A) and anthocyanin (B). The colors of anthocyanidins depend in part on the substituents attached to ring B (see Table 1). An increase in the number of hydroxyl groups shifts absorption to a longer wavelength and gives a bluer color. Replacement of a hydroxyl group with a methoxyl group (OCH_3) shifts absorption to a slightly shorter wavelength, resulting in a redder color.

Anthocyanin color is influenced by many factors, including the number of hydroxyl and methoxyl groups in ring B of the anthocyanidin (see Figure 8A), the presence of aromatic acids esterified to the main skeleton, and the pH of the cell vacuole in which these compounds are stored.

Anthocyanins may also exist in supramolecular complexes along with chelated metal ions and flavone copigments. The blue pigment of dayflower (*Commelina communis*) was found to consist of a large complex of six anthocyanin molecules, six flavones, and two associated magnesium ions The most common anthocyanidins and their colors are shown in Figure 8 and Table 1.

TABLE 1
Effects of ring substituents on anthocyanidin color

Anthocyanidin	Substituents	Color
Pelargonidin	4′— OH	Orange red
Cyanidin	3′— OH, 4′— OH	Purplish red
Delphinidin	3′— OH,4′— OH,5′— OH	Bluish purple
Peonidin	3′— OCH_3, 4′— OH	Rosy red
Petunidin	3′— OCH_3, 4′— OH, 5′— OCH_3	Purple

Considering the variety of factors affecting anthocyanin coloration and the possible presence of carotenoids as well, it is not surprising that so many different shades of flower and fruit color are found in nature. The evolution of flower color may have been governed by selection pressures for different sorts of pollinators, which often have different color preferences.

5.2. Flavonoids May Protect against Damage by Ultraviolet Light

Two other major groups of flavonoids found in flowers are **flavones** and **flavonols** (see Figure 5).

These flavonoids generally absorb light at shorter wavelengths than anthocyanins do, so they are not visible to the human eye.

However, insects such as bees, which see farther into the ultraviolet range of the spectrum than humans do, may respond to flavones and flavonols as attractant cues.

Flavonols in a flower often form symmetric patterns of stripes, spots, or concentric circles called *nectar guides*. These patterns may be conspicuous to insects and are thought to help indicate the location of pollen and nectar.

Flavones and flavonols are not restricted to flowers; they are also present in the leaves of all green plants. These two classes of flavonoids function to protect cells from excessive UV-B radiation (280–320 nm) because they accumulate in the epidermal layers of leaves and stems and absorb light strongly in the UV-B region while allowing the visible (photosynthetically active) wavelengths to pass through uninterrupted. In addition, exposure of plants to increased UV-B light has been demonstrated to increase the synthesis of flavones and flavonols.

Arabidopsis thaliana mutants that lack the enzyme **chalcone synthase** produce no flavonoids. Lacking flavonoids, these plants are much more sensitive to UV-B radiation than wild-type individuals are, and they grow very poorly under normal conditions. When shielded from UV light, however, they grow normally. A group of simple phenylpropanoid esters are also important in UV protection in *Arabidopsis*.

Other functions of flavonoids have recently been discovered. For example, **flavones and flavonols secreted into the soil by legume roots mediate the interaction of legumes and nitrogen-fixing symbionts.** Recent work suggests that flavonoids also play a regulatory role in plant development as modulators of **polar auxin transport**.

5.3 Isoflavonoids Have Antimicrobial Activity

The **isoflavonoids** (isoflavones) are a group of flavonoids in which the position of one aromatic ring (ring B) is shifted (see Figure 5).

Isoflavonoids are found mostly in legumes and have several different biological activities. Some, such as the rotenoids, have strong insecticidal actions; others have anti-estrogenic effects.

For example, sheep grazing on clover rich in isoflavonoids often suffer from infertility. The isoflavonoid ring system has a three-dimensional structure similar to that of steroids (see Figure 3B), allowing these substances to bind to estrogen receptors.

Isoflavonoids may also be responsible for the anticancer benefits of food prepared from soybeans. In the past few years, isoflavonoids have become best known for their role as *phytoalexins*, antimicrobial compounds synthesized in response to bacterial or fungal infection that help limit the spread of the invading pathogen.

6 Tannins Deter Feeding by Herbivores

A second category of plant phenolic polymers with defensive properties, besides lignins, is the **tannins**. The term *tannin* was first used to describe compounds that could convert raw animal hides into leather in the process known as tanning. Tannins bind the collagen proteins of animal hides, increasing their resistance to heat, water, and microbes. There are two categories of tannins: condensed and hydrolyzable.

Condensed tannins are compounds formed by the polymerization of flavonoid units. They are frequent constituents of woody plants. Because condensed tannins can often be hydrolyzed to anthocyanidins by treatment with strong acids, they are sometimes called *pro-anthocyanidins*.

Hydrolyzable tannins are heterogeneous polymers containing phenolic acids, especially gallic acid, and simple sugars. They are smaller than condensed tannins and may be hydrolyzed more easily; only dilute acid is needed. Most tannins have molecular masses between 600 and 3000.

Tannins are general toxins that significantly reduce the growth and survivorship of many herbivores when added to their diets. In addition, tannins act as feeding repellents to a great diversity of animals. Mammals such as cattle, deer, and apes characteristically avoid plants or parts of plants with high tannin contents. Unripe fruits, for instance, frequently have very high tannin levels, which may be concentrated in the outer cell layers.

Interestingly, humans often prefer a certain level of astringency in tannin-containing foods, such as apples, blackberries, tea, and red wine.

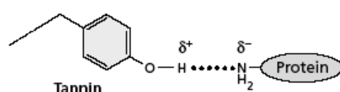
Recently, polyphenols (tannins) in red wine were shown to block the formation of **endothelin-1**, a signaling molecule that makes blood vessels constrict. This effect of wine tannins may account for the often-touted health benefits of red wine, especially the reduction in the risk of heart disease associated with moderate red wine consumption.

Although moderate amounts of specific polyphenolics may have health benefits for humans, the defensive properties of most tannins are due to their toxicity, which is generally attributed to their ability to bind proteins nonspecifically. It has long been thought ***that plant tannins complex proteins in the guts of herbivores by forming hydrogen bonds between their hydroxyl groups and electronegative sites on the protein*** (Figure 9A).

More recent evidence indicates that tannins and other phenolics can also bind to dietary protein in a covalent fashion (see Figure 9B). The foliage of many plants contains enzymes that oxidize phenolics to their corresponding quinone forms in the guts of herbivores.

Quinones are highly reactive electrophilic molecules that readily react with the nucleophilic —NH_2 and —SH groups of proteins (see Figure 9B). By whatever mechanism protein–tannin binding occurs, this process has a negative impact on herbivore nutrition. Tannins can inactivate herbivore digestive enzymes and create complex aggregates of tannins and plant proteins that are difficult to digest.

(A) Hydrogen bonding between tannins and protein



(B) Covalent bonding to protein after oxidation

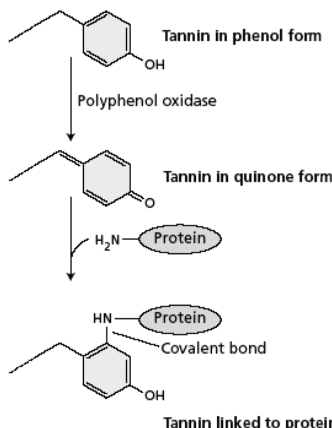


FIGURE 9 Proposed mechanisms for the interaction of tannins with proteins. (A) Hydrogen bonds may form between the phenolic hydroxyl groups of tannins and electronegative sites on the protein. (B) Phenolic hydroxyl groups may bind covalently to proteins following activation by oxidative enzymes, such as polyphenol oxidase.

Herbivores that habitually feed on tannin-rich plant material appear to possess some interesting adaptations to remove tannins from their digestive systems. For example, some mammals, such as rodents and rabbits, produce salivary proteins with a very high proline content (25–45%) that have a high affinity for tannins. Secretion of these proteins is induced by ingestion of food with a high tannin content and greatly diminishes the toxic effects of tannins. The large number of proline residues gives these proteins a very flexible, open conformation and a high degree of hydrophobicity that facilitates binding to tannins.

Plant tannins also serve as defenses against microorganisms. For example, the nonliving heartwood of many trees contains high concentrations of tannins that help prevent fungal and bacterial decay.

III. NITROGEN-CONTAINING COMPOUNDS

A large variety of plant secondary metabolites have nitrogen in their structure. Included in this category are such well-known antiherbivore defenses as alkaloids and cyanogenic glycosides, which are of considerable interest because of their toxicity to humans and their medicinal properties. Most nitrogenous secondary metabolites are biosynthesized from common amino acids.

In this section we will examine the structure and biological properties of various nitrogen-containing secondary metabolites, including **alkaloids, cyanogenic glycosides, glucosinolates, and nonprotein amino acids.**

In addition, we will discuss the ability of *systemin*, a protein released from damaged cells, to serve as a wound signal to the rest of the plant.

1. Alkaloids Have Dramatic Physiological Effects on Animals

The **alkaloids** are a large family of more than 15,000 nitrogen- containing secondary metabolites found in approximately 20% of the species of vascular plants. The nitrogen atom in these substances is usually part of a **heterocyclic ring**, a ring that contains both nitrogen and carbon atoms.

As a group, alkaloids are best known for their striking pharmacological effects on vertebrate animals. As their name would suggest, most alkaloids are alkaline. At pH values commonly found in the cytosol (pH 7.2) or the vacuole (pH 5 to 6), the nitrogen atom is protonated; hence, alkaloids are positively charged and are generally water soluble.

TABLE 2
Major types of alkaloids, their amino acid precursors, and well-known examples of each type

Alkaloid class	Structure	Biosynthetic precursor	Examples	Human uses
Pyrrolidine		Ornithine (aspartate)	Nicotine	Stimulant, depressant, tranquilizer
Tropane		Ornithine	Atropine	Prevention of intestinal spasms, antidote to other poisons, dilation of pupils for examination
			Cocaine	Stimulant of the central nervous system, local anesthetic
Piperidine		Lysine (or acetate)	Coniine	Poison (paralyzes motor neurons)
Pyrrolizidine		Ornithine	Retrorsine	None
Quinolizidine		Lysine	Lupinine	Restoration of heart rhythm
Isoquinoline		Tyrosine	Codeine	Analgesic (pain relief), treatment of coughs
			Morphine	Analgesic
Indole		Tryptophan	Psilocybin	Halucinogen
			Reserpine	Treatment of hypertension, treatment of psychoses
			Strychnine	Rat poison, treatment of eye disorders

Alkaloids are usually synthesized from one of a few common amino acids—in particular, lysine, tyrosine, and tryptophan. However, the carbon skeleton of some alkaloids contains a component derived from the terpene pathway. Table 2 lists the major alkaloid types and their amino acid precursors.

Several different types, including nicotine and its relatives, are derived from ornithine, an intermediate in arginine biosynthesis.

The B vitamin nicotinic acid (niacin) is a precursor of the pyridine (six-membered) ring of this alkaloid; the pyrrolidine (fivemembered) ring of nicotine arises from ornithine (Figure 13.18). Nicotinic acid is also a constituent of NAD+ and NADP+, which serve as electron carriers in metabolism.

The role of alkaloids in plants has been a subject of speculation for at least 100 years. Alkaloids were once thought to be nitrogenous wastes (analogous to urea and uric acid in animals), nitrogen storage compounds, or growth regulators, but there is little evidence to support any of these functions.

Most alkaloids are now believed to function as defenses against predators, especially mammals, because of their general toxicity and deterrence capability.

Large numbers of livestock deaths are caused by the ingestion of alkaloid-containing plants. In the United States, a significant percentage of all grazing livestock animals are poisoned each year by consumption of large quantities of alkaloid-containing plants such as lupines (*Lupinus*), larkspur (*Delphinium*), and groundsel (*Senecio*).

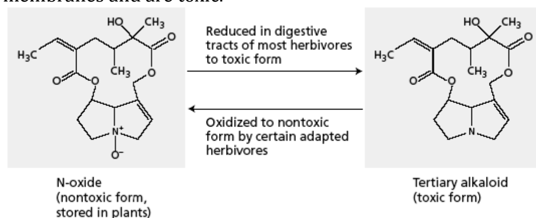
This phenomenon may be due to the fact that domestic animals, unlike wild animals, have not been subjected to natural selection for the avoidance of toxic plants. Indeed, some livestock actually seem to prefer alkaloid containing plants to less harmful forage.

Nearly all alkaloids are also toxic to humans when taken in sufficient quantity. For example, **strychnine, atropine, and coniine** (from poison hemlock) are classic alkaloid poisoning agents. At lower doses, however, many are useful pharmacologically. Morphine, codeine, and scopolamine are just a few of the plant alkaloids currently used in medicine.

Other alkaloids, including **cocaine, nicotine, and caffeine**, enjoy widespread nonmedical use as stimulants or sedatives.

On a cellular level, the mode of action of alkaloids in animals is quite variable. Many alkaloids interfere with components of the nervous system, especially the chemical transmitters; others affect membrane transport, protein synthesis, or miscellaneous enzyme activities.

One group of alkaloids, the pyrrolizidine alkaloids, illustrates how herbivores can become adapted to tolerate plant defensive substances and even use them in their own defense. Within plants, pyrrolizidine alkaloids occur naturally as nontoxic N-oxides. In herbivore digestive tracts, however, they are quickly reduced to uncharged, hydrophobic tertiary alkaloids, which easily pass through membranes and are toxic.



Nevertheless, some herbivores, such as cinnabar moth (*Tyria jacobeae*), have developed the ability to reconvert tertiary pyrrolizidine alkaloids to the nontoxic N-oxide form immediately after its absorption from the digestive tract. These herbivores may then store the N-oxides in their bodies as defenses against their own predators.

Not all of the alkaloids that appear in plants are produced by the plant itself. Many grasses harbor endogenous fungal symbionts that grow in the apoplast and synthesize a variety of different types of alkaloids.

Grasses with fungal symbionts often grow faster and are better defended against insect and mammalian herbivores than those without symbionts.

Unfortunately, certain grasses with symbionts, such as tall fescue, are important pasture grasses that may become toxic to livestock when their alkaloid content is too high.

Like monoterpenes in conifer resin and many other antiherbivore defense compounds, alkaloids increase in response to initial herbivore damage, fortifying the plant against subsequent attack.

For example, *Nicotiana attenuata,* a wild tobacco that grows in the deserts of the Great Basin, produces higher levels of nicotine following herbivory. When it is attacked by nicotine- tolerant caterpillars, however, there is no increase in nicotine. Instead, volatile terpenes are released that attract enemies of the caterpillars. Clearly, wild tobacco and other plants must have ways of determining what type of herbivore is damaging their foliage.

Herbivores might signal their presence by the type of damage they inflict or the distinctive chemical compounds they release.

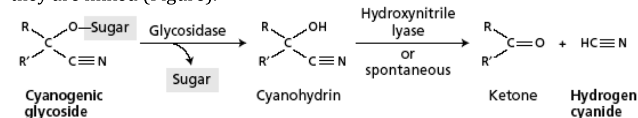
Recently, the oral secretions of caterpillars feeding on corn leaves were shown to contain a fatty acid–amino acid conjugate that induced the plant to produce defensive terpenes when applied to cut leaves.

2. Cyanogenic Glycosides Release the Poison Hydrogen Cyanide

Various nitrogenous protective compounds other than alkaloids are found in plants. Two groups of these substances— cyanogenic glycosides and glucosinolates—are not in themselves toxic but are readily broken down to give off volatile poisons when the plant is crushed. Cyanogenic glycosides release the well-known poisonous gas hydrogen cyanide (HCN).

The breakdown of cyanogenic glycosides in plants is a two-step enzymatic process. Species that make cyanogenic glycosides also make the enzymes necessary to hydrolyze the sugar and liberate HCN:

1. In the first step the sugar is cleaved by a glycosidase, an enzyme that separates sugars from other molecules to which they are linked (Figure).



2. In the second step the resulting hydrolysis product, called an α-hydroxynitrile or cyanohydrin, can decompose spontaneously at a low rate to liberate HCN. This second step can be accelerated by the enzyme hydroxynitrile lyase.

Cyanogenic glycosides are not normally broken down in the intact plant because the glycoside and the degradative enzymes are spatially separated, in different cellular compartments or in different tissues. In sorghum, for example, the cyanogenic glycoside dhurrin is present in the vacuoles of epidermal cells, while the hydrolytic and lytic enzymes are found in the mesophyll. Under ordinary conditions this compartmentation prevents decomposition of the glycoside. When the leaf is damaged, however, as during herbivore feeding, the cell contents of different tissues mix and HCN forms.

Cyanogenic glycosides are widely distributed in the plant kingdom and are frequently encountered in legumes, grasses, and species of the rose family. Considerable evidence indicates that cyanogenic glycosides have a protective function in certain plants. HCN is a fast-acting toxin that inhibits metalloproteins, such as the iron-

containing cytochrome oxidase, a key enzyme of mitochondrial respiration. The presence of cyanogenic glycosides deters feeding by insects and other herbivores, such as snails and slugs. As with other classes of secondary metabolites, however, some herbivores have adapted to feed on cyanogenic plants and can tolerate large doses of HCN. The tubers of cassava (*Manihot esculenta*), a high-carbohydrate, staple food in many tropical countries, contain high levels of cyanogenic glycosides. Traditional processing methods, such as grating, grinding, soaking, and drying, lead to the removal or degradation of a large fraction of the cyanogenic glycosides present in cassava tubers.

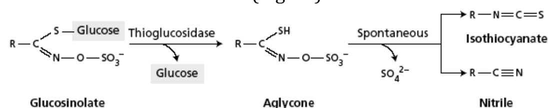
However, chronic cyanide poisoning leading to partial paralysis of the limbs is still widespread in regions where cassava is a major food source because the traditional detoxification methods employed to remove cyanogenic glycosides from cassava are not completely effective. In addition, many populations that consume cassava have poor nutrition, which aggravates the effects of the cyanogenic glycosides.

Efforts are currently under way to reduce the cyanogenic glycoside content of cassava through both conventional breeding and genetic engineering approaches. However, the complete elimination of cyanogenic glycosides may not be desirable because these substances are probably responsible for the fact that cassava can be stored for very long periods of time without being attacked by pests.

3. Glucosinolates Release Volatile Toxins

A second class of plant glycosides, called the **glucosinolates**, or mustard oil glycosides, break down to release volatile defensive substances. Found principally in the Brassicaceae and related plant families, glucosinolates give off the compounds responsible for the smell and taste of vegetables such as cabbage, broccoli, and radishes.

The release of these mustard-smelling volatiles from glucosinolates is catalyzed by a hydrolytic enzyme, called a thioglucosidase or myrosinase, that cleaves glucose from its bond with the sulfur atom (Figure).



The resulting aglycone, the nonsugar portion of the molecule, rearranges with loss of the sulfate to give pungent and chemically reactive products, including isothiocyanates and nitriles, depending on the conditions of hydrolysis.

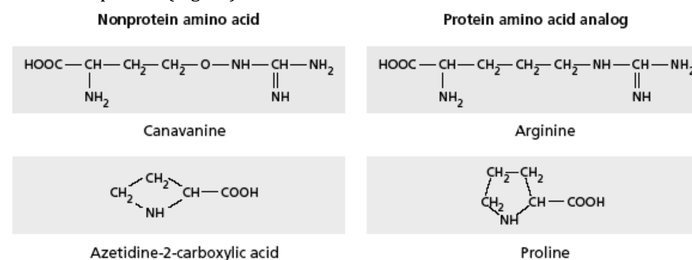
These products function in defense as herbivore toxins and feeding repellents. Like cyanogenic glycosides, glucosinolates are stored in the intact plant separately from the enzymes that hydrolyze them, and they are brought into contact with these enzymes only when the plant is crushed.

As with other secondary metabolites, certain animals are adapted to feed on glucosinolate-containing plants without ill effects. For adapted herbivores, such as the cabbage butterfly, glucosinolates often serve as stimulants for feeding and egg laying, and the isothiocyanates produced after glucosinolate hydrolysis act as volatile attractants.

Most of the recent research on glucosinolates in plant defense has concentrated on rape, or canola (*Brassica napus*), a major oil crop in both North America and Europe. Plant breeders have tried to lower the glucosinolate levels of rapeseed so that the high-protein seed meal remaining after oil extraction can be used as animal food. The first low-glucosinolate varieties tested in the field were unable to survive because of severe pest problems. However, more recently developed varieties with low glucosinolate levels in seeds but high glucosinolate levels in leaves are able to hold their own against pests and still provide a protein-rich seed residue for animal feeding.

4. Nonprotein Amino Acids Defend against Herbivores

Plants and animals incorporate the same 20 amino acids into their proteins. However, many plants also contain unusual amino acids, called **nonprotein amino acids**, that are not incorporated into proteins but are present instead in the free form and act as protective substances. Nonprotein amino acids are often very similar to common protein amino acids. **Canavanine**, for example, is a close analog of arginine, and azetidine-2-carboxylic acid has a structure very much like that of proline (Figure).



Nonprotein amino acids exert their toxicity in various ways. Some block the synthesis or uptake of protein amino acids; others, such as **canavanine**, can be mistakenly incorporated into proteins. After ingestion, canavanine is recognized by the herbivore enzyme that normally binds arginine to the arginine transfer RNA molecule, so it becomes incorporated into proteins in place of arginine. The usual result is a nonfunctional protein because either its tertiary structure or its catalytic site is disrupted. Canavanine is less basic than arginine and may alter the ability of an enzyme to bind substrates or catalyze chemical reactions.

Plants that synthesize nonprotein amino acids are not susceptible to the toxicity of these compounds. The jack bean (*Canavalia ensiformis*), which synthesizes large amounts of canavanine in its seeds, has protein-synthesizing machinery that can discriminate between canavanine and arginine, and it does not incorporate canavanine into its own proteins. Some insects that specialize on plants containing nonprotein amino acids have similar biochemical adaptations.

4.1 Some Plant Proteins Inhibit Herbivore Digestion

Among the diverse components of plant defense arsenals are proteins that interfere with herbivore digestion. For example, some legumes synthesize α-amylase inhibitors that block the action of the starch-digesting enzyme α-amylase. Other plant species produce **lectins**, defensive proteins that bind to carbohydrates or carbohydrate-containing proteins.

After being ingested by an herbivore, lectins bind to the epithelial cells lining the digestive tract and interfere with nutrient absorption .

The best-known antidigestive proteins in plants are the proteinase inhibitors. Found in legumes, tomatoes, and other

plants, these substances block the action of herbivore proteolytic enzymes. After entering the herbivore's digestive tract, they hinder protein digestion by binding tightly and specifically to the active site of protein-hydrolyzing enzymes such as trypsin and chymotrypsin.

Insects that feed on plants containing proteinase inhibitors suffer reduced rates of growth and development that can be offset by supplemental amino acids in their diet.

The defensive role of proteinase inhibitors has been confirmed by experiments with transgenic tobacco. Plants that had been transformed to accumulate increased levels of proteinase inhibitors suffered less damage from insect herbivores than did untransformed control plants.

4.2 Herbivore Damage Triggers a Complex Signaling Pathway

Proteinase inhibitors and certain other defenses are not continuously present in plants, but are synthesized only after initial herbivore or pathogen attack. In tomatoes, insect feeding leads to the rapid accumulation of proteinase inhibitors throughout the plant, even in undamaged areas far from the initial feeding site.

The systemic production of proteinase inhibitors in young tomato plants is triggered by a complex sequence of events:

- Wounded tomato leaves synthesize **prosystemin**, a large (200 amino acid) precursor protein.
- Prosystemin is proteolytically processed to produce the short (18 amino acid) polypeptide called **systemin**, the first (and so far only) polypeptide hormone discovered in plants (Pearce et al. 1991) (Figure 9).
- Systemin is released from damaged cells into the apoplast.
- Systemin is then transported out of the wounded leaf via the phloem.
- In target cells, systemin is believed to bind to a site on the plasma membrane and initiate the biosynthesis of **jasmonic acid**, a plant growth regulator that has wide-ranging effects (Creelman and Mullet 1997).
- Jasmonic acid eventually activates the expression of genes that encode proteinase inhibitors (see Figure 9). Other signals, such as ABA (abscisic acid), salicylic acid, and pectin fragments from damaged plant cell walls also appear to participate in this wound signaling cascade, but their specific roles are still unclear.

4.4 Jasmonic Acid Is a Plant Stress Hormone That Activates Many Defense Responses

Jasmonic acid levels rise steeply in response to damage caused by a variety of different herbivores and trigger the formation of many different kinds of plant defenses besides proteinase inhibitors, including terpenes and alkaloids.

The structure and biosynthesis of jasmonic acid have intrigued plant biologists because of the parallels to some eicosanoids that are central to inflammatory responses and other physiological processes in mammals.

In plants, jasmonic acid is synthesized from linolenic acid (18:3), which is released from membrane lipids and then converted to jasmonic acid.

Jasmonic acid is known to induce the transcription of a host of genes involved in plant defense metabolism. The mechanisms for this gene activation are slowly becoming clear.

For example, recent research on the Madagascar periwinkle (*Catharanthus roseus*), which makes some valuable anticancer alkaloids, identified a transcription factor that responds to jasmonic acid by activating the expression of several genes encoding alkaloid biosynthetic genes.

Interestingly, this transcription factor also activates the genes of certain primary metabolic pathways that provide precursors for alkaloid formation, so it appears to be a master regulator of metabolism in Madagascar periwinkle.

Direct demonstration of the role of jasmonic acid in insect resistance has come from research with mutant lines of *Arabidopsis* that produce only low levels of jasmonic acid. Such mutants are easily killed by insect pests, such as fungus gnats, that normally do not damage *Arabidopsis*. However, application of exogenous jasmonic acid can restore resistance nearly to the levels of the wild-type plant.

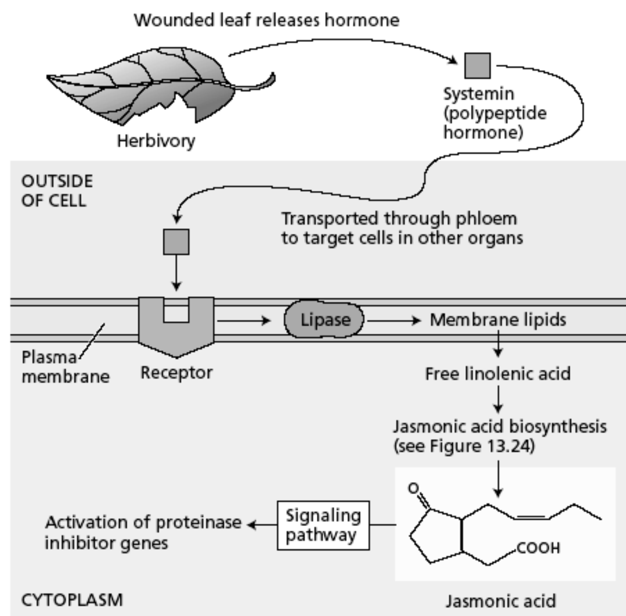


FIGURE 9 Proposed signaling pathway for the rapid induction of proteinase inhibitor biosynthesis in wounded tomato plants.

PLANT DEFENSE AGAINST PATHOGENS

Even though they lack an immune system, plants are surprisingly resistant to diseases caused by the fungi, bacteria, viruses, and nematodes that are ever present in the environment. In this section we will examine the diverse array of mechanisms that plants have evolved to resist infection, including the production of antimicrobial agents and a type of programmed cell death called the *hypersensitive response*. Finally, we will discuss a special type of plant immunity called *systemic acquired resistance*.

1. Some Antimicrobial Compounds Are Synthesized before Pathogen Attack

Several classes of secondary metabolites that we have already discussed have strong antimicrobial activity when tested in vitro; thus they have been proposed to function as defenses against pathogens in the intact plant. Among these are saponins, a group of triterpenes thought to disrupt fungal membranes by binding to sterols.

Experiments performed in the laboratory of Anne Osbourn at the John Innes Centre (Norwich, England) utilized genetic approaches to demonstrate the role of saponins in defense against pathogens of oat. Mutant oat lines with reduced saponin levels had much less resistance to fungal pathogens than wild-type oats. Interestingly, one fungal strain that normally grows on oats was able to detoxify one of the principal saponins in the plant. However, mutants of this strain that could no longer detoxify saponins failed to infect oats, but could grow successfully on wheat that did not contain any saponins.

2. Infection Induces Additional Antipathogen Defenses

Some defenses are induced by herbivore attack or microbial infection. Defenses that are produced only after initial herbivore damage theoretically require a smaller investment of plant resources than defenses that are always present, but they must be activated quickly to be effective. Like proteinase inhibitors, other induced defenses appear to be triggered by complex signal transduction networks, which often involve jasmonic acid.

After being infected by a pathogen, plants deploy a broad spectrum of defenses against invading microbes. A common defense is the **hypersensitive response**, in which cells immediately surrounding the infection site die rapidly, depriving the pathogen of nutrients and preventing its spread. After a successful hypersensitive response, a small region of dead tissue is left at the site of the attempted invasion, but the rest of the plant is unaffected.

The hypersensitive response is often preceded by the production of **reactive oxygen species**. Cells in the vicinity of the infection synthesize a burst of toxic compounds formed by the reduction of molecular oxygen, including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH).

An NADPH-dependent oxidase located on the plasma membrane (Figure 10) is thought to produce O_2^-, which in turn is converted to ·OH and H_2O_2. The hydroxyl radical is the strongest oxidant of these active oxygen species and can initiate radical chain reactions with a range of organic molecules, leading to lipid peroxidation, enzyme inactivation, and nucleic acid degradation.

Active oxygen species may contribute to cell death as part of the hypersensitive response or act to kill the pathogen directly.

Many species react to fungal or bacterial invasion by synthesizing lignin or callose. These polymers are thought to serve as barriers, walling off the pathogen from the rest of the plant and physically blocking its spread.

A related response is the modification of cell wall proteins. Certain proline-rich proteins of the wall become oxidatively cross-linked after pathogen attack in an H_2O_2-mediated reaction (see Figure 10). This process strengthens the walls of the cells in the vicinity of the infection site, increasing their resistance to microbial digestion.

Another defensive response to infection is the formation of hydrolytic enzymes that attack the cell wall of the pathogen. An assortment of glucanases, chitinases, and other hydrolases are induced by fungal invasion. Chitin, a polymer of *N*-acetylglucosamine residues, is a principal component of fungal cell walls.

These hydrolytic enzymes belong to a group of proteins that are closely associated with pathogen infection and so are known as **pathogenesis- related** (**PR**) **proteins**.

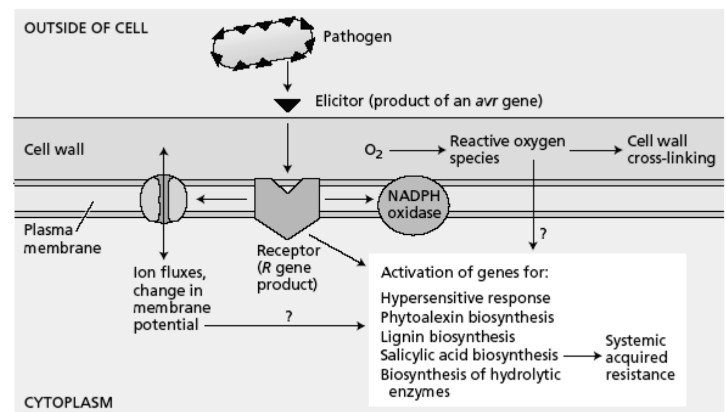


FIGURE 10 Many modes of antipathogen defense are induced by infection. Fragments of pathogen molecules called elicitors initiate a complex signaling pathway leading to the activation of defensive responses. Some bacterial protein elicitors are injected directly into the cell, where they interact with *R* gene products.

3. Phytoalexins.

Perhaps the best-studied response of plants to bacterial or fungal invasion is the synthesis of **phytoalexins**. Phytoalexins are a chemically diverse group of secondary metabolites with strong antimicrobial activity that accumulate around the site of infection. Phytoalexin production appears to be a common mechanism of resistance to pathogenic microbes in a wide range of plants. However, different plant families employ different types of secondary products as phytoalexins.

For example, isoflavonoids are common phytoalexins in the legume family, whereas in plants of the potato family (Solanaceae), such as potato, tobacco, and tomato, various sesquiterpenes are produced as phytoalexins.

Phytoalexins are generally undetectable in the plant before infection, but they are synthesized very rapidly after microbial attack because of the activation of new

biosynthetic pathways. The point of control is usually the initiation of gene transcription. Thus, plants do not appear to store any of the enzymatic machinery required for phytoalexin synthesis. Instead, soon after microbial invasion they begin transcribing and translating the appropriate mRNAs and synthesizing the enzymes de novo.

Although phytoalexins accumulate in concentrations that have been shown to be toxic to pathogens in bioassays, the defensive significance of these compounds in the intact plant is not fully known. Recent experiments on genetically modified plants and pathogens have provided the first direct proof of phytoalexin function in vivo.

For example, when tobacco was transformed with a gene catalyzing the biosynthesis of the phenylpropanoid phytoalexin resveratrol, it became much more resistant to a fungal pathogen than non-transformed control plants were. In contrast, *Arabidopsis* mutants deficient in the tryptophan-derived phytoalexin camalexin were more susceptible than the wildtype to a fungal pathogen. In other experiments, pathogens that had been transformed with genes encoding phytoalexindegrading enzymes were then able to infect plants that were normally resistant to them.

4. Some Plants Recognize Specific Substances Released from Pathogens

Within a species, individual plants often differ greatly in their resistance to microbial pathogens. These differences often lie in the speed and intensity of a plant's reactions. Resistant plants respond more rapidly and more vigorously to pathogens than susceptible plants. Hence it is important to learn how plants sense the presence of pathogens and initiate defense.

In the last few years, researchers have isolated over 20 different plant resistance genes, known as ***R* genes**, that function in defense against fungi, bacteria, and nematodes. Most of the *R* genes are thought to encode protein receptors that recognize and bind specific molecules originating from pathogens. This binding alerts the plant to the pathogen's presence (see Figure 10). The specific pathogen molecules recognized are referred to as **elicitors**, and they include proteins, peptides, sterols, and polysaccharide fragments arising from the pathogen cell wall, outer membrane, or a secretion process.

The *R* gene products themselves are nearly all proteins with a leucine-rich domain that is repeated inexactly several times in the amino acid sequence.

Such domains may be involved in elicitor binding and pathogen recognition. In addition, the *R* gene product is equipped to initiate signaling pathways that activate the various modes of antipathogen defense. Some *R* genes encode a nucleotide-binding site that binds ATP or GTP; others encode a protein kinase domain.

R gene products are distributed in more than one place in the cell. Some appear to be situated on the outside of the plasma membrane, where they could rapidly detect elicitors; others are cytoplasmic to detect either pathogen molecules that are injected into the cell or other metabolic changes indicating pathogen infection. *R* genes constitute one of the largest gene families in plants and are often clustered together in the genome. The structures of *R* gene clusters may help generate *R* gene diversity by promoting exchange between chromosomes.

Studies of plant disease have revealed complex patterns of host relationships between plants and pathogen strains. Plant species are generally susceptible to the attack of certain pathogen strains, but resistant to others. This specificity is thought to be determined by interaction between the products of host *R* genes and pathogen ***avr*** (***avir*ulence) genes** believed to encode specific elicitors. According to current thinking, successful resistance requires the elicitor, a product of the pathogen *avr* gene, to be rapidly recognized by a host plant receptor, the product of an *R* gene. Despite their name, *avr* genes appear to encode factors that promote infection.

5. Exposure to Elicitors Induces a Signal Transduction Cascade

Within a few minutes after pathogen elicitors have been recognized by an R gene, complex signaling pathways are set in motion that lead eventually to defense responses (see Figure 10).

A common early element of these cascades is a transient change in the ion permeability of the plasma membrane. *R* gene activation stimulates an influx of Ca^{2+} and H^{+} ions into the cell and an efflux of K^{+} and Cl^{-} ions.

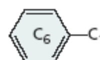
The influx of Ca^{2+} activates the oxidative burst that may act directly in defense, as well as signaling other defense reactions. Other components of pathogen-stimulated signal transduction pathways include nitric oxide, mitogen-activated protein (MAP) kinases, calcium-dependent protein kinases, jasmonic acid, and salicylic acid (see the next section).

6. A Single Encounter with a Pathogen May Increase Resistance to Future Attacks

When a plant survives the infection of a pathogen at one site, it often develops increased resistance to subsequent attacks at sites throughout the plant and enjoys protection against a wide range of pathogen species.

This phenomenon, called **systemic acquired resistance** (**SAR**), develops over a period of several days following initial infection (Ryals et al. 1996). Systemic acquired resistance appears to result from increased levels of certain defense compounds that we have already mentioned, including chitinases and other hydrolytic enzymes.

Although the mechanism of SAR induction is still unknown, one of the endogenous signals is likely to be **salicylic acid**. The level of this benzoic acid derivative,



a compound rises dramatically in the zone of infection after initial attack, and it is thought to establish SAR in other parts of the plant, although salicylic acid itself is not the mobile signal.

In addition to salicylic acid, recent studies suggest that its methyl ester, methyl salicylate, acts as a volatile SAR inducing signal transmitted to distant parts of the plant and even to neighboring plants. Thus, even though plants lack immune systems like those present in many animals, they have developed elaborate mechanisms to protect themselves from disease-causing microbes.

H. Stress physiology: Responses of plants to abiotic (water, temperature and salt) stresses; mechanisms of tolerance to abiotic stress

1. External conditions that adversely affect growth, development, or productivity are termed as stress.

2. Stresses trigger a wide range of plant responses such as
- altered gene expression
- cellular metabolism
- changes in growth rates and crop yields

3. Stress may be broadly categorized into two groups
- **Biotic -** imposed by other organisms
- **Abiotic -** arising from an excess or deficit in the physical or chemical environment

4. Biotic and abiotic stresses can reduce average plant productivity by 65% to 87%, depending on the crop.

PLANT RESPONSE TO ABIOTIC STRESS

5. Environmental conditions that can cause stress are water-logging, drought, high or low temperatures, excessive soil salinity, inadequate mineral in the soil, too much or too little light and phytotoxic compounds such as Ozone

6. Resistance or sensitivity of plants to stress depends **on the species, the genotype and development age**

7. Stress resistance mechanisms
- Avoidance mechanisms: prevents exposure to stress
- Tolerance mechanisms: permit the plant to withstand stress
- Acclimation: alter their physiology in response stress

8. Regulation of plant stress responses is mediated by **Abscisic acid (ABA), Jasmonic acid, Ethylene** and **Calcium**

9. Changes in gene expression to stress
- A stress response is initiated when plants recognizes stress at the cellular level
- Stress recognition activates signal transduction pathways that transmit information within the individual cell and throughout the plant
- Changes in gene expression may modify growth and development and even influence reproductive capabilities

10. Gene expression results in:
- Increase amounts of specific mRNA
- Enhance translation
- Stabilize proteins
- Altered protein activity
- A combination of the above

11. Stresses involving water deficit: Water related stresses could affect plants if the environment contains insufficient water to meet basic needs

12. Environmental conditions that can lead to water deficit are drought, hypersaline conditions, low temperatures and transient loss of turgor at midday

13. Factors that can affect the response of a plant to water deficit
- duration of water deficiency
- the rate of onset
- if the plant was acclimated to water stress

14. Tolerance to drought and salinity
- **Osmotic adjustment:** a biochemical mechanism that helps plants acclimate to dry and saline conditions
- Many drought-tolerant plants can regulate their solute potentials to compensate for transient or extended periods of water stress by making osmotic adjustments, which results in a net increase in the number of solutes particles present in the plant cell
- Osmotic adjustment occurs when the concentrations of solutes within a plant cell increases to maintain a positive turgor pressure within the cell
- The cell actively accumulates solutes and as a result the solute potential (Ψs) drops, promoting the flow of water into the cell

15. Compatible solutes that contribute to osmotic adjustments: **Proline, Glycine betanine,β-alanine betanine, Dimethyl sulphonioproponate, Proline betanine, Choline-O-sufate, mannitol and pinnitol**

16. **Compatible solutes (osmolytes)**: tend to be neutrally charged at physiological pH, either non-ionic or zwitterionic, and are excluded from hydration shells of macromolecules.
- In contrast, many ions can enter the hydration shells of a protein and promote its denaturation
- Membrane-associated carriers and transporters are probably involved in differentially distributing osmolytes within the cell and throughout the plant.

17. Proline: distribution of proline in osmotically stressed plants involves a transporter. Two *Arabidopsis* cDNA encoding proline transporters were cloned by functional complementation

18. Glycine Betanine: Glycine betaine accumulation in osmotically stressed plants resulted from increased rates of synthesis, whereas, with proline, synthesis and catabolism appears to be co-ordinately regulated in response to water stress
- Glycine betaine is synthesised and accumulated by many algae and higher plants and is not broken down by plants
- Genetic evidence indicates that accumulation of glycine betaine promotes salt tolerance

19. Mannitol is the reduced form of the sugar mannose and is broadly distributed among plants.
- salt stress inhibits sucrose synthesis and promotes accumulation of mannitol
- mannitol concentrations increase in response to osmotic stress
- mannitol accumulation appears to be regulated by competing pathways and decreasing rates of mannitol consumption and catabolism
- In celery, salt stress inhibits sucrose synthesis but does not seem to affect the enzymes that synthesise mannitol

- Salt stress also down-regulate NAD^+ -dependent mannitol dehydrogenase, the enzyme that oxidises mannitol in celery
- Transgenic tobacco and *Arabidopsis* plants engineered to express the *E. coli* gene for NAD+-dependent mannitol-1-phosphate dehydrogenase, which converts fructose 6-phosphate to mannitol-1-phosphate, synthesised mannitol, although at low concentrations. In transgenic tobacco salt tolerance was improved, and seeds able to germinate in the presence of salt

20. D-Pinitol : cyclic sugar alcohol, is a major solute in members of the Pine Family and Bean Family.

- its concentrations are higher among halophytic species and those adapted to drought.
- in leaves, pinitol is localised to the chloroplast and cystol but not in the vacuoles

21. Osmotin is an abundant alkaline protein discovered in cultured tobacco cells that had been acclimated to 428 mM NaCl. Its molecular size of 26-kDa

- localised in the vacuole
- classified as a pathogenesis-related (PR) protein
- Transcription of an osmotin gene is induced by at least 10 signals:
- ABA, ethylene, auxin, infection by TMV, salinity, lack of water, cold, UV light, wounding, and fungal infection
- Many of the stress-induced genes are regulated by ABA
- ABA plays a role in stomata closure and induction of gene expression

22. Oxidative Stress: Oxidative stress results from conditions promoting the formation of active oxygen species that damage or kill cells

23. Environmental factors that cause oxidative stress are air pollution (increased amounts of ozone or sulfur dioxide), oxidant forming herbicides e.g. paraquat dichloride, heavy metals, drought, heat and cold stress, wounding, UV light and intense light that stimulate **photoinhibition**

24. Reactive oxygen species (ROS): Formed during certain redox reactions and during incomplete reduction of oxygen or oxidation of water by the mitochondrial or chloroplast electron transfer chain. Examples: Singlet oxygen, hydrogen peroxide, superoxide anion, hydroxyl and perhydroxyl radicals

25. Ozone and oxidative stress: Hydrocarbons and oxides of nitrogen (NO, NO_2) and sulfur (SO_x) react with solar UV radiation to generate ozone (O_3). Ozone is a highly reactive oxidant

26. The negative effects of ozone on plants

- decreased rates of photosynthesis
- leaf injury
- reduced growth of shoots and roots
- accelerated senescence
- reduced crop yield

27. Ozone alters ion transport, increases membrane permeability, inhibits H^+-pump activity, collapses membrane potential, increases Ca^{2+} uptake from the apoplasm and Oxidative damage to biomolecules

28. Resistance to ozone: Utilizes either **avoidance** or **tolerance**

- Avoidance involves physically excluding the pollutant by closing the stomata, the principal site at which ozone enters the plant
- Tolerance - biochemical responses that induce or activate the antioxidant defence system and possibly also various repair mechanisms

29. Tolerance to oxidative stress

Antioxidant or antioxidant enzyme	Stress condition
Anionic peroxidases	Chilling, high CO_2
Ascorbate peroxidase	Drought, high CO_2, high light intensity, ozone, paraquat
Catalase	Chilling
Glutathione	Chilling, drought, γ-irradiation, heat stress, high CO_2, ozone, SO_2
Glutatione reductase	Chilling, drought, high CO_2, ozone, paraquat
Polyamines	Deficiency of K, P, Ca, Mg, Mn, S, or B; drought, heat, ozone
Superoxide dismutase	Chilling, high CO2, high light, increased O_2, ozone, paraquat, SO_2

30. Salicylic acid and ethylene: Ozone exposure results in increased amounts of H_2O_2, which stimulate the production of SA. This results in a transient increase in the number of transcripts that **encode defence-related secondary metabolites** e.g. phytoalexins, cellular barrier molecules e.g. lignins, callose, and extensins, PR proteins e.g. (1→3) β-glucanase, chitinase, gluthatione *S*-transferase and phenylalanine ammonia lyase. Increases ethylene production by inducing increases in ACC synthase and ACC oxidase gene transcription

31. Heat Stress: The typical response to heat stress is a decrease in the synthesis of normal proteins, accompanied by an accelerated transcription and translation of new proteins known as **heat shock proteins** (HSPs)

32. Heat shock may arise in leaves when transpiration is insufficient or when stomata are partially or fully closed and irradiance is high or may arise in germinating seedlings when the soil is warmed by the sun and even may arise in organs with reduced capacity for transpiration e.g. fruits

33. Resistance or sensitivity of plants to heat stress depends on Duration, Severity of the stress, susceptibility of different cell types and Stage of development

34. Classes of Heat Shock Proteins (HSPs)

Protein class	Size (kDa)	Location
HSP100	100-114	cytoplasm
HSP90	80-94	cytoplasm, ER
HSP70	69-71	ER, cytoplasm, mitochondria
HSP60	10-60	chloroplasts, mitochondria
smHSP	15-30	cytoplasm, chloroplast, ER, mitochondria

PLANT RESPONSE TO BIOTIC STRESS

1. Pathogen attack strategies

- **necrotrophy**, in which the plant cells are killed
- **biotrophy**, in which the plant cells remain alive
- **hemibiotrophy**, in which the pathogen initially keeps cells alive but kills them at later stages of infection

2. Cause of failure of a pathogen to cause disease

- The plant species attacked is unable to support the life-strategy of the particular pathogen
- The plant possesses preformed structural barriers or toxic compounds
- Defence mechanisms are activated such that the invasion remains localized

- Environmental conditions change and the pathogen perish

3. Successful pathogen infection and disease occurs:

- Only if the environmental conditions are favourable
- If the preformed plant disease defenses are inadequate
- If the plant fail to detect the pathogen
- If activated defense responses are ineffective

4. Preformed defense is present in form of Secondary metabolites

- Plants possess different secondary metabolites with antimicrobial properties
- may be present in their biological active form or may be store as inactive precursors that are converted to their active forms by host enzymes in response to pathogen attack or tissue damage

5. Secondary metabolites: pre-formed inhibitors are the **saponins** and the **glucosinolates.**

- Saponins are glycosylated compounds, classified as either triterpinoids, steroids, or steroidal glycoalkaloids.
- A biologically active triterpinoid saponin found in the roots of oat plants, **avenacin A-1**, is highly effective against the root infecting fungus, a major pathogen of cereal roots.
- This pathogen affects wheat and barley, but not oat plants due to avencin A1.

6. Hypersensitive response

- 1st line of activated defence, occurs within 24hr
- Recognition of a genetically incompatible pathogen
- Creates unfavorable conditions for pathogen growth and reproduction
- Impair the spread of harmful enzymes and toxins
- Leads to localised cell and tissue death

7. Reactive oxygen species (ROS): the production of ROS such as superoxide and hydrogen peroxide (H_2O_2) is often the first response detected, occurring within 5 min on pathogen infection. The mechanism plants have for producing superoxide from molecular oxygen probably involves a plasma membrane-associated NADPH oxidase

8. Role of ROS in plant defence

- H_2O_2 may be directly toxic to pathogens
- may contribute to the structural reinforcement of plant cell walls, either by cross-linking various hydroxyproline and proline rich glycoprotein to the polysaccharide matrix or by increasing the rate of lignin polymer formation by way of peroxidase enzyme activity
- make the plant cell wall more resistant to microbial perpetration and enzymatic degradation
- H_2O_2 induces benzoic acid 2 hydrolase (BA 2-H) enzyme activity, which is required for biosynthesis of Salicylic Acid.
- H_2O_2 is known to induce genes for proteins involved in certain cell protection mechanisms e.g. glutathione *S*-transferase

9. Nitric oxide synthesis (NO): In plants, rapid *de novo* synthesis of nitric oxide accompanies the recognition of avirulent pathogenic bacteria.

- Nitric Oxide has the capacity to potentiate induction of plant cell death by ROS.
- Nitric Oxide is known to bind heme and could inhibit catalase and ascorbate peroxidase, which detoxifies H_2O_2
- In the presence of inhibitors of Nitric oxide production, the HR diminishes, disease symptoms become more severe, and bacterial growth is increased
- Nitric oxide and Reactive Oxygen Speceis play an important synergistic role in the rapid activation of a wide repertoire of defence responses after pathogen attack

10. Benzoic acid and salicylic acid: Both SA and BA are derived from the phenyl-propanoid pathway and have many roles in plant defence responses. Accumulate to high concentrations in the vicinity of incompatible infection sites

11. Jasmonic acid: Jasmonic acid (JA) is an oxylipin-like hormone derived from oxygenated linolenic acid.

- Increases in JA in response to pathogen/insect attack occur both locally and systematically
- Spraying methyl-JA onto plants increases their resistance to some (but not all) necrotrophic fungi, but not to biotrophic fungi or bacteria

12. Ethylene: Ethylene is frequently synthesised during both incompatible and compatible interactions

- Ethylene is required to mediate both resistance against necrotrophic fungal pathogens and against soil borne fungal species that are not ordinarily plant pathogens
- Ethylene and JA are required for activation of proteinase inhibitor (PI) genes and certain PR and chitinase genes

13. Pathogenesis-related (PR) proteins include fungal cell wall-degrading enzymes, chitinases, glucanases, lipoxygenase, anti-microbial polypeptides and components of signal transduction cascades.

- Salicylic Acid-mediated signal transduction cascades regulate the transcriptional activation of many Pathogenesis Related genes
- Ethylene and SA have been shown to act synergistically, further enhancing the expression of PR genes

14. Plant defensins: Type of defence-related genes with demonstrated antimicrobial activity codes small (<7 kDa) cysteine-rich peptides that accumulate in the periphery of the plant plasma membrane. This is frequently found in dry plant seeds. Induction of the defensin *PDF1-2* gene transcript require ethylene or Me-jasmonate

15. Pytoalexins: Low-molecular-mass, lipophilic antimicrobial compounds that accumulate rapidly at sites of incompatible pathogen infection.

- Biosynthesis occurs only after primary metabolic precursors are diverted into a novel secondary metabolic pathway e.g. phenylalanine is diverted into the synthesis of various flavonoid phytoalexins by the de novo synthesis of phenylalanine ammonia lyase (PAL).

16. Systemic plant defence responses

- Defence responses elaborated in tissues far from the invasion site and even in neighbouring plants
- The type of response is determined by the attacking organism

- Responses to fungi, bacteria, and viruses are distinct from the response to insect
- Nematodes appear to induce a mixture of responses
- Root colonising non-pathogenic bacteria induce another type of response

17. Systemic acquired resistance (SAR) : Fungi, bacteria, and viruses activate systemically a specific subset of PR-type gene by a mechanism known as **systemic acquired resistance** (SAR) in which local necrosis formation at the initial site of pathogen invasion triggers both a local increase in SA accumulation and the formation of a phloem-mobile signal

- For SAR to occur, the initial infection must result in formation of necrotic lesions, either as part of the HR or as symptom of disease
- SA concentrations increase and volatile methyl-SA is released in distal plant tissue
- PR proteins in the non-invaded parts of the plants are synthesised resulting in reduction in disease symptoms after subsequent infection of many pathogenic species

18. Induced systemic resistance (ISR):

- Non-pathogenic root-colonizing rhizobacteria cause induced system resistance
- Rhizobacteria that promote specific plant growth, for example *P. fluorescens*, induce a systemic resistance response that does not depend on SA or PR protein accumulation
- Instead, ISR requires both JA and ethylene signalling and also the SAR regulatory protein NPRI