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Review

Curriculum Vitae

Toshiyuki Nagata, born on January 9, 1945, Nagano Prefecture, Japan

Education

1968	B.Sc. University of Tokyo
1973	Ph.D. University of Tokyo

Professional experience

1973–1979	University of Tokyo
1979–1983	Nagoya University
1983–1990	Associate Professor, National Institute of Basic Biology
1990–2007	Professor, University of Tokyo
2007	Professor Emeritus, University of Tokyo
2007	Professor, Hosei University
2008 to date	Professor and Dean, Faculty of Bioscience and Applied Chemistry, Hosei University
1974–1975, 1976, 1983	Alexander von Humboldt Fellow, Max-Planck-Institut für Biologie, Tübingen, Germany
1980–1982	Member of US-Japan Scientific Collaboration Program on Plant Biotechnology
1995–2001, 2005–2007	Director of the Botanical Gardens, University of Tokyo

Honors

1997	Alexander von Humboldt Research Award
1998	Associate Member of the EMBO
2005	Research Award of Botanical Society of Japan
2006	Research Award of Japanese Society of Plant Cell and Molecular Biology

A Journey with Plant Cell Division: Reflection at My Halfway Stop

Toshiyuki Nagata

Contents

Abstract I have studied various aspects of plant cell division for more than 40 years, and here I try to illustrate some foci among them. First, establishment of the induction of cell division in freshly isolated protoplasts from leaves is described, as this is important not only for biotechnology, but also for the cell cycle transition from G_0 to S phase, whose importance has not been fully understood yet. The role of plant hormones, auxin and cytokinin, on this process has not been fully elucidated. Secondly, establishment of a model plant cell system of tobacco BY-2 cells and the high level of cell cycle synchrony achieved using this system is also an important theme for me. However, I will not go into details, but instead refer to the two

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recently published monographs edited by myself (Nagata T, Hasezawa S, Inzé D, Biotechnology in agriculture and forestry, vol. 53, Tobacco BY-2 Cells. Springer, Berlin, 2004; Nagata T, Matsuoka K, Inzé D, Biotechnology in agriculture and forestry, vol. 58. Tobacco BY-2 cells. From cellular dynamics to omics, Springer, Berlin, 2006). Further, habituation, discovered by Gautheret (Bull Soc Chim Biol 24:13–47, 1942), will be described, and I will describe how recently we have identified key molecules that may play an important role in this process. Finally, I will try to illustrate aspects of heterophylly from our studies on *Ludwigia arcuata* (Onagraceae). Although heterophylly is thought to be mostly related to cell elongation in leaves and induced by environmental cues, we found that in *L. arcuata,* it is closely associated with cell division. This system offers an opportunity to understand the role of cell division upon leaf shape determination as affected by environmental cues. Thus, as my scientific issues are always associated with cell division, their aspects and background stories will be described in this chapter.

1 Introduction

The title of this chapter may sound a bit strange. It originates from the following fact: I retired from the University of Tokyo in the spring of 2007 at the age of 62 and became a professor emeritus, as the age limitation of Japanese national universities is rather strict! So, when I was asked to write a review reflecting my past, I thought this may be a good time to reflect what I have tried to do in research. However, I am newly appointed as a full professor at Hosei University, a kind of ivy league university in Tokyo and further, I am appointed as Dean of the Faculty of Bioscience and Applied Chemistry, which started in the Spring of 2008. Although currently, I am strongly involved in administration, I wish to extend what I have done before. So, I remain in science and to this aim, I am preparing for this new start!

When I was first asked to write this chapter, the editor simply asked me to write a kind of reflective account of my work with tobacco BY-2 cells that spans more than 27 years of research. However, I have published two monographs on this subject recently (Nagata et al. 2004, 2006) and many colleagues contributed to these two volumes, so I did not think that reiteration of those reviews would be very worthwhile. Subsequently, the editor told me that this chapter could be redirected towards some specific aspect of plant genetics. One of the distinguished contributors to Progress in Botany's special reviews was Professor Diter von Wettstein (2006), whom I know very well. Hence, I was particularly honored to be given the opportunity of contributing in the same way. I decided to write this review with greater emphasis placed on my studies of tobacco mesophyll protoplasts, as there are a certain numbers of "hidden stories" on this topic.

I have sought answers to the problem of cell division events throughout my career. However, I have to confess I feel uneasy when challenged by new problems and, as such, this review mainly reflects how I have been pulled towards the control of cell division in plants; like a magnet.

2 Attempts to Culture Protoplasts

I like to start with culture of tobacco mesophyll protoplasts. This is partly because this relates to my first published study but this is also related to an important issue of cell cycle transition, from G_0 to S phase, in which there are still many unresolved issues. In fact, my academic career started with culturing tobacco mesophyll protoplasts, which became major parts of my Ph.D. thesis at the University of Tokyo. Perhaps, I should tell what was behind the start of this work. When I graduated from the University of Tokyo in 1968, it coincided with a year of massive student discontent and social unrest in Japan; I refer to it as "the university struggle." Actually the university graduation ceremony for the academic year of 1968 was canceled because of this struggle. Then, I was enrolled in the graduate school there in the Spring of 1968. Although I captured basic techniques on how to handle plant cells according to plant cell and tissue techniques under the supervision of Professor Toshio Yamaki, there was much less enthusiasm on how these techniques could be directed to key scientific issues. Nonetheless, I established a partial cell synchrony system using the tobacco XD6 cell line. After auxin-starvation of the cells in auxin-free medium, I could see that the addition of auxin to these auxin-depleted cells induced a partial cell synchrony. Although I never published this result, this experience was useful for inducing auxin-induced cell synchrony in the auxin-starved tobacco BY-2 cells more than 20 years later (Ishida et al. 1993).

Because of the chaotic situation on the Tokyo campus, I sought some sanctuary. So I asked Dr. Itaru Takebe, Governmental Institute for Plant Virus Research, Chiba, to join his group as a kind of voluntary student. They had just published a methodology for the preparation of protoplasts from tobacco leaves (Takebe et al. 1968) and their successful infection with tobacco mosaic virus (TMV) (Takebe and Otsuki 1969). Although I had several speculative ideas, one of my initial intentions was to isolate subcellular fractions and to examine the effects of plant hormones on these components. Needless to say, protoplasts are single naked cells in the strict sense and it was intriguing to work with this novel material. When I visited Dr. Takebe, he told me that a reliable technique for making protoplasts from leaves had not been established in any cultural conditions and that this was an urgent task to be resolved. This sounded a reasonable challenge for a young student, so I decided immediately to establish suitable culture conditions for preparing protoplasts from tobacco mesophyll. It took a month or so to establish conditions for preparing enzymes that could function effectively in aseptic conditions, as at that time, such techniques had not been well established. Further, most of the works had to be done in a normal laboratory condition that lacked a laminar flow chamber. Thus, most work had to be done on a Saturday, as it was quiet with nobody around to infect the cultures! Within a month, I established protoplasts from tobacco mesophyll aseptically but they died quickly. Most of possible media were tried in which some protoplasts survived for a week or so but no cell division was observed apart from sporadic endomitosis. This situation lasted for 3–4 months.

Culture of somatic cells including leaf cells was first proposed by Gottlieb Haberlandt (1902), but the real start of plant cell and tissue culture was much later. After the discovery of the plant hormone, auxin, basic disciplines of plant tissue culture started through studies of Philip R. White (1939) and Roger Gautheret (1939). However, culture of cells directly prepared from leaves was far behind. By 1968, there were two cases, in which cell division was observed in mesophyll cells directly isolated from leaves. Kohlenbach (1960) reported cell division in mesophyll cells of *Macleaya cordata* (Papaveraceae), while Joshi and Noggle (1967) reported successful culture of isolated leaf cells of peanut (*Arachis hypogaea*). Generally speaking, however, leaf cells were not considered to be suitable for culturing. This is well reflected in a rather comprehensive study by Joshi and Ball (1968), in which they described mechanical separation of mesophyll cells from several species and their trials to culture them. They concluded that primarily, mechanical isolation of mesophyll cells from leaves was very difficult even if their division was observed in culture. Such a case was peanut, where cell division was observed only in adjacent cells and but never in freely suspended cells. Referring to a rather negative description for the culture of leaf cells even in the second edition of the standard monograph of "The cultivation of animal and plant cells" by White (1964), they also discussed difficulties in culturing mesophyll cells, such as palisade and spongy parenchyma cells. They concluded that failure to culture mesophyll cells from leaves was because of the complete differentiated state of these cells and that meristematic cells may offer the only feasible start point for leaf cell culture in vitro. Thus, the consensus view was that it was impossible! However, I persisted with this problem and I recall a laboratory members' excursion to Oku-Nikko, ca. 100 km from the city of Tokyo on 2 November 1969. Just before the trip, I made a fresh preparation of tobacco mesophyll protoplasts. When I returned on November 5, I suddenly observed the first induced cell division. It was not sporadic, but most cells divided in a week or so, which was reproducible. What was the difference between this successful case and previous unsuccessful ones? Previously our material was tobacco cultivar Bright Yellow, a common cultivar in Japan. After unsuccessful experiments with this material, I tried to make protoplasts from another cultivar Xanthi nc, a model system for studying TMV infection and local lesion assays. Such a simple change of cultivar choice was a complete surprise. However, the more I thought about it, it became increasingly clear that the quality of the starting material and the need for it to be fresh and grown under optimal conditions became increasingly important criteria for guaranteeing the success of protoplast culture. This success countered the worries expressed by Joshi and Ball (1968) that isolation of mesophyll cells was very difficult. Indeed my results gave me confidence that leaf protoplast culture could be applied more widely.

In addition to emphasizing these critical preparative points, I dared to add another seemingly unusual observation. Good protoplasts which have mitotic competence and well-able to form colonies always have a tendency to show clear "blinking" or "sparkling" of grana in their chloroplasts when viewed under blight field illumination. If blinking of grana is weak, healthy protoplasts cannot be

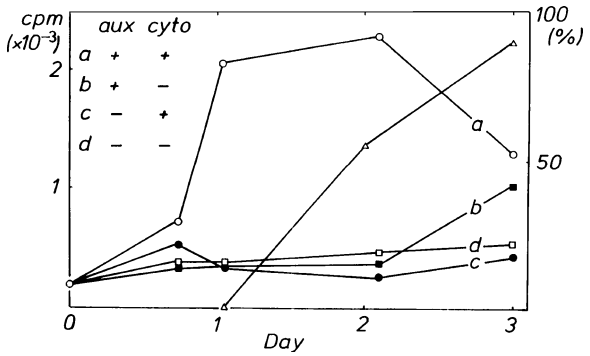

Fig. 1 Time course of DNA synthesis in cultured tobacco mesophyll protoplasts. Cell division was observed only in both presence of auxin and cytokinin. Although DNA synthesis was observed in the sole presence of cytokinin, this DNA synthesis did not result in the completion of cell cycle. DNA synthesis was measured by the incorporation of ^{3}H-thymidine into acid-insoluble fraction; however, this DNA synthesis was also confirmed by the incorporation of BrdU into nucleus, which was examined by staining with an antibody against BrdU (Shimizu and Nagata, unpublished observations)

obtained. Although I tried to explain this on numerous occasions and indeed demonstrated to others in the laboratory, it seems to have gone unnoticed in more recent protocols for protoplast preparation. So we published our findings, but we did not realize at the time that Nagata and Takebe (1970) would be listed as Citation Classics by the Institute for Scientific Information (ISI) in 1988.

In our work, high frequency of cell division was induced in tobacco mesophyll protoplasts in a rather simple medium supplemented with auxin and cytokinin under white light illumination (Nagata and Takebe 1970); this straightforward approach is not considered too much by others working in this field. In fact, DNA synthesis was detected as early as 12 h after protoplast isolation and the first cell division was detected by the second day of culture under optimal condition (Fig. 1). In a week, most protoplasts divided at least once. Regarding this issue, there have been published several related published articles (Zelcer and Galun 1976; Meyer et al. 1984). However, in other studies, start of DNA synthesis and cell division was much slower and nonsynchronous. Only according to our original protocol, can such rapid induction of DNA synthesis and cell division be observed. For sustained cell division, further development of our techniques was required, which will be described below.

Immediately after that, regeneration of whole plants from single protoplasts of tobacco mesophyll was shown using a revised medium in which high frequency of the induction of cell division in respective leaf protoplasts was shown (Nagata and Takebe 1971). In particular, when protoplasts were embedded in an agar medium, a high frequency of colony formation was confirmed unambiguously. Although we did not emphasize this point in writing the paper, another interesting phenomenon was observed. It was clear that colony formation was completely dependent on cell density (ca. 1,000 ml^{-1}) that was a threshold marker for forming colonies. This is

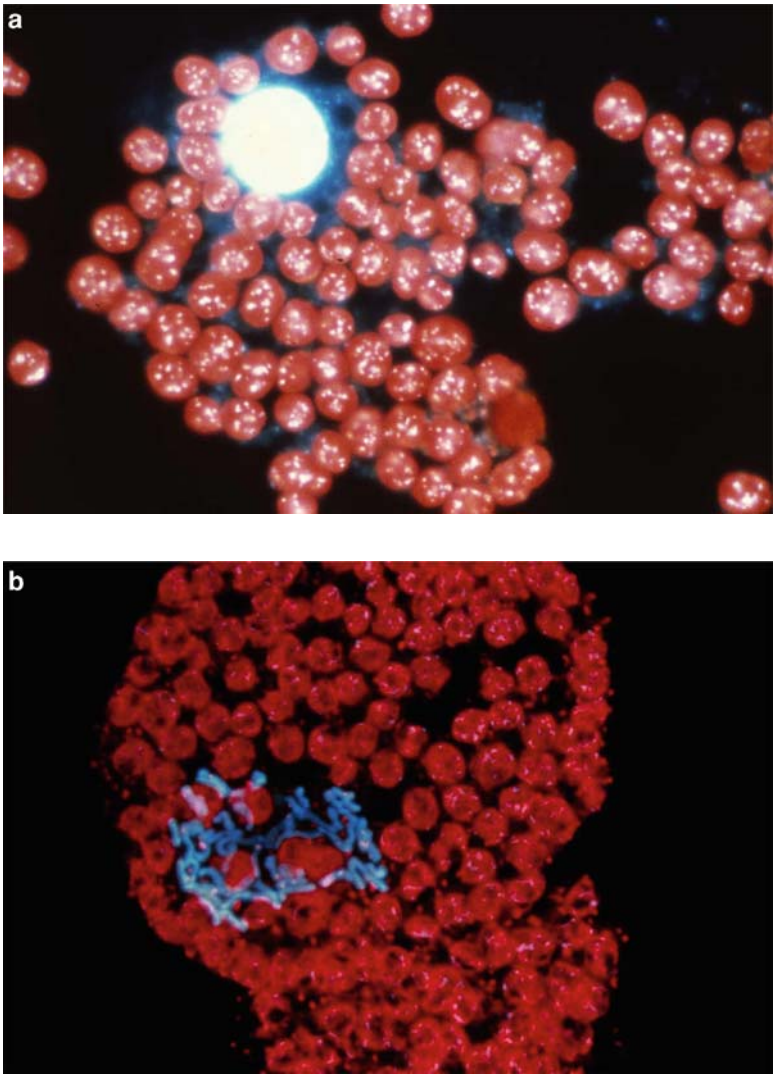

Fig. 2 Strict dependency of colony formation of plated tobacco mesophyll protoplasts on cell density. (**a**) When tobacco mesophyll protoplasts were embedded in the agar medium, colony formation was observed at higher than 1,000 per ml. (**b**) Embedding of the protoplasts in the agar medium was carried out on the agar medium of the same composition (bottom layer). When the thickness of embedded agar medium was made in a gradient from left to right, colony formation was only observed in the left half of the plated medium. Although in the right half, cells were embedded in the agar medium, no colony was observed. This may be explained by the leakage of the factors that were produced by cells to the bottom layer so that level of the factors necessary for colony formation became lower, resulting in the abortion of colony formation. This factor may be something-like Phytosulfokine as described by Matsubayashi and Sakagami (1996)

reflected in Fig. 2, as colony formation was observed above a threshold density, but never below it. We thought that there might be an enrichment of mitogenic factors that would be reaching optimal levels to facilitate cell division of leaf protoplasts. We intended to identify these mitogenic factors, but our facility was not sufficient to resolve anything at the biochemical level. Many years later, when I read a paper on phytosulfokine (PSK) by Matsubayashi and Sakagami (1996), I thought this could have been at least part of our perceived mitogenic stimulus. Our paper (Nagata and Takebe 1971) was also selected in Citation Classics by ISI (1985), in which we demonstrated that totipotency of plant somatic cells in tissues is nearly 100%. In a seminal paper by Steward et al. (1963), totipotency of plant cells was demonstrated, but the induction of cell division in somatic cells was shown to be only at ca. 5–8% (Israel and Steward 1966). This poses a question about the remaining 92–95% of cells in the somatic tissues. In contrast, we have shown that cell division can be induced in almost all cells in leaf tissue at the optimal condition. So in the essay added to the Citation Classics (1985), I wrote explicitly that this is the real start of somatic cell genetics and totipotency of plant cells.

3 Interim Period or Wandering Years

3.1 Germany

After receiving a Ph.D. from the University of Tokyo in 1973, I was appointed as an assistant professor in the Department of Pure and Applied Sciences of the University of Tokyo. Immediately after that, Professor Georg Melchers at the Max Planck Institute for Biology invited me to work in his laboratory in Tübingen, Germany and I went there as an Alexander von Humboldt Fellow. At the time, I proposed a project on transformation of protoplasts with *Agrobacterium tumefaciens*, as the transformation of plant cells should be a next target for our adventure. Actually, shortly before my departure to Germany, I read with great interest a paper by Professor Jeff Schell and others (Van Larebeke et al. 1974), in which Ti plasmid was identified as the causative agent for crown gall disease. However, because of the general interests in the laboratory and available facilities, Professor Melchers asked me to do work on cell fusion. So I did a kind of theoretical analysis on protoplast fusion in relation to cell–cell interactions (Nagata and Melchers 1978; Nagata and Nagata 1984a). In fact, I measured surface charges of protoplasts by cell electrophoresis using an apparatus made by a technician in the laboratory using an old cathetometer that Professor Carl Correns had used. Around that time, I also found that polyvinyl alcohol of low molecular mass is an agent for inducing cell fusion (Nagata 1978), just like polyethylene glycol whose effect was discovered by Kao and Michayluk (1974).

I returned to Japan in 1975, but I went back and forth to Tübingen and some other places in Germany until the passing away of Professor Melchers on November 22, 1998. He was an extraordinary man and trusted me to the extent that he called me his "fourth son." Somewhat later, Professor Fritz Melchers, his eldest son and former Director of the Basel Institute of Immunology, visited me and told me that although his father had many guests and coworkers in his laboratory, I was the only one that his father called his son. So from that moment, Fritz and his two other brothers and I became a kind of quasibrotherhood. Around a year before he died, Prof G. Melchers sent me a whole set of his publications with a letter saying that if some day he would die, I would be asked to write an obituary. Even after his death, I have been in Tübingen several times and in 2006, on the anniversary of what would have been his 100th birthday, I attended a symposium in his honor where Professor Detlef Weigel gave a plenary lecture on "florigen." This was timely because the florigen concept was first proposed by three scientists; Melchers, Chailakhyan, Kuiper in 1937. For almost 70 years, the biochemical identity of florigen remained elusive but at last was proven to be a product of the *FT* gene (Turck et al. 2008). So, I wrote a kind of obituary to Professor Melchers together with reporting this new finding in a Japanese journal on Heredity (Iden in Japanese).

3.2 Enzymes

As noted in the beginning, my scientific carrier started with culture of protoplasts from tissues, and knowledge about enzymes became obligatory. As may be well known, enzymes that are used for the preparation of protoplasts have been mostly and are still produced by Japanese manufacturers. The enzymes that have been used for this purpose were found first by Dr. Takebe (Takebe et al. 1968); Macerozyme R10 and Cellulose Onozuka R10 (Yakult Pharmaceutical Co. Tokyo). When I was working at the Max Planck Institute for Biology in 1976, I knew a Japanese researcher Dr. S. Ishii of Kikkoman Co, who had reported a novel type of pectin-degrading enzyme, pectin lyase, based on the information from Chemical Abstract. I visited him after I returned to Japan. The enzyme named Pectolyase from *Aspergillus japonicus*, which is used for a fermentation process of soya source, was found to be very much powerful for degrading plant tissues. This is most likely to be due to the presence of both *endo*-pectin lyase and *endo*-polygalacturonase (Ishii 1976). However, the initial sample that I obtained from Dr. Ishii was very toxic to plant cells. After a partial purification by Dr. Ishii, it became useful for preparing protoplasts from a wide variety of plant tissues. Particularly with this enzyme when combined with cellulose, rapid preparation of protoplasts became possible. I tried to persuade Kikkoman Co. to produce and sell this enzyme but the initial reply was negative. However, a paper was published by Nagata and Ishii (1979) using an enzyme mixture of Pectolyase and cellulose; DuPont Co. ordered it from Kikkoman Co. without even asking the price and the company the decided to

produce and sell it under the trade name of Pectolyase Y23 through Seishin Pharmaceutical Co., a subsidiary of Kikkoman Co.

Another example is Cellulose Onozuka RS. From 1978 to 1981, I used protoplasts prepared from *Catharanthus roseus* cultured cells, which are easily produced by using a combination of Pectolyase Y23 and Cellulose Onozuka R10. When we started to use tobacco BY-2 cells, something rather odd occurred. A graduate student was working with tobacco BY-2 cells and was studying the infection of protoplasts with TMV and was almost able to perfect the technique of protoplasts infection with TMV, which was later published by Kikkawa et al. (1982). However, on one occasion, he told me that the infection technique was no longer reproducible. When I looked through his whole experimental procedure and my experimental notes, it turned out that successful infection of the protoplasts with TMV was only achieved with a kind of probe enzyme material that the manufacturer, Yakult Pharmaceutical Co., offered us. When I asked them what was the difference between the probe material and the commercially available one, they replied that it was produced from a mutated strain of *Trichoderma viride* by UV irradiation. It was also revealed that the enzyme prepared from the mutated *T. viride* actually contained a wider spectrum of enzymes including hemicellulose. In fact, with this enzyme cocktail, protoplasts could be prepared from various plant species. Again we asked the manufacturer to produce it and it became available under the brand name of Cellulose Onozuka RS by the Yakult Pharmaceutical Co. (Nagata et al. 1981). Since then, I have had a close relationship with these manufacturers and from time to time am asked to examine the quality of their enzymes.

3.3 Move to Nagoya

In 1979, I moved to Nagoya University. In fact, Dr. Takebe was offered with a full professor position at Nagoya University. A prerequisite for him to accept it was that I could go with him to Nagoya as he did not have much experience at the university. So I said yes and moved to Nagoya. Our intention was to establish a delivery system of genetic materials into protoplasts by any means. To this end, first we did liposome-mediated gene delivery into protoplasts prepared from *C. roseus* (Fukunaga et al. 1981). This was demonstrated more clearly by using tobacco BY-2 cells (Nagata et al. 1981). I worked for more than 27 years with tobacco BY-2 cells and have accumulated a lot of information on this line. However, as mentioned in the Introduction, stories of experiments we have done on the tobacco BY-2 cell will not be described here (see Nagata et al. 2004, 2006).

Our main aim was to establish systems to transform protoplasts with *A. tumefaciens* or Ti plasmid. Because of our poor facility at that time, Ti plasmid was offered by Professors Milt Gordon and Nene Nester, University of Washington, Seattle, USA. However, before our success with this trial, a Dutch group reported a series of their successful trials (Márton et al. 1979).

3.4 Miles International Symposium

In May 1982, I was invited to give a talk at the 14th Miles International Symposium at Johns Hopkins University, Baltimore, USA (Nagata 1984b). This meeting was unusual and extremely interesting; animal, plant and microbial scientists assembled to discuss cell fusion and transformation techniques and their potentials. Here, I learned about the frontiers of animal cell transformation, which was a very competitive field at the time. One unforgettable meeting was with Professor Jeff Schell, Max Planck Institute for Plant Breeding, Cologne, Germany, although I knew about him through a series of seminal works. Since then, we developed a close scientific relationship and we met at many occasions in many locations until his untimely death in 2002; later, he recommended me as an associate member of the EMBO in 1998.

3.5 Move to Okazaki

In December 1983, I moved to Okazaki, as an associate professor at the interuniversity facility of the National Institute for Basic Biology. I worked in the Cell Biology Division headed by Professor Tsuneyoshi Kuroiwa, who was enthusiastic to see molecules including DNA under the microscope. He developed a very sensitive DNA detection system using fluorescence microscopy on samples stained with 4′,6-diamidino-2-phenylindole (DAPI). Thus, I learnt how to detect traces of DNA upon fertilization in plants by DAPI staining. Although maternal inheritance was discovered by Carl Correns and was confirmed in many organisms, molecular

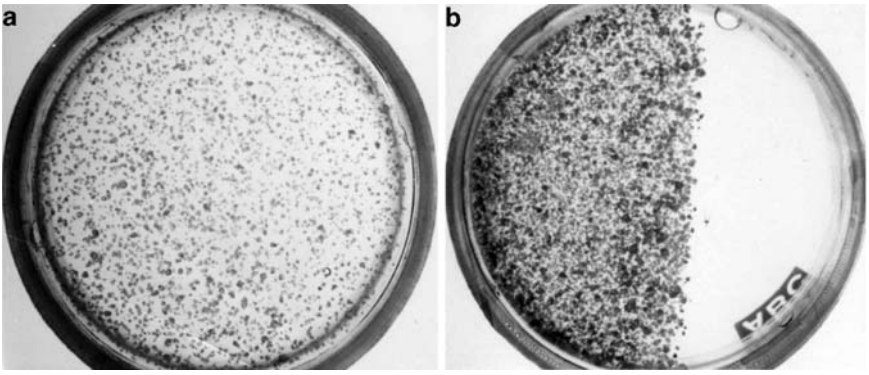

Fig. 3 Fluorescence microscopy of tobacco mesophyll protoplasts after staining with DAPI. (**a**) A freshly isolated protoplast. Whereas location of DNA in nucleus and chloroplasts was clearly demonstrated, DNA was also observed in mitochondria. Note that red fluorescence in chloroplasts is originated from chlorophyll. (**b**) Protoplasts culture for 36 h. When nuclear division was observed, red fluorescence in chloroplasts became darker

proof for this was insufficient. Kuroiwa et al. (1984) provided molecular proof for maternal inheritance in *Chlamydomonas reinhardtii*. By using these techniques, we examined the fate of organelle genomes upon fertilization in various plant species (Miyamura et al. 1986, 1987). Unexpectedly, Professor Lloyd Morgensen (Northern Arizona University) wrote to me that our work was the first clear proof of this phenomenon. I also followed the fate of early stages of cultured tobacco mesophyll protoplasts after staining with DAPI (Fig. 3), which clearly demonstrated morphological changes to the protoplasts at the subcellular level.

4 Settlement at Tokyo

I was appointed as a full professor at the University of Tokyo in 1990 and worked there until my retirement in 2007. During this period, my first focus was on the establishment of tobacco BY-2 cells as the model cell line of higher plants, on which I refer here only to the points that have not been included previously (Nagata et al. 2004, 2006). Secondly, I tried to understand the molecular basis of the early events that occur when tobacco mesophyll protoplasts undergo G_0 to S phase transition. In particular, I studied the effects of auxin and cytokinin on this process. Thirdly, I devoted time to understand habituation, whose molecular basis has not been clarified yet and which remains the most enigmatic phenomenon in plant cell culture even since its discovery by Gautheret (1942). Further, I spent time studying heterophylly, which is an ecophysiological phenomenon but is a good system to understand the relationship between an environmental cue and leaf morphogenesis. At the University of Tokyo, I was also involved in administration of the Botanical Gardens as a director for 8 years and did some work on biodiversity, but space does not permit any description of this work here.

4.1 Tobacco BY-2 Cells

When I arrived to Tokyo, the first task was to describe details on the handling of tobacco BY-2 cells. We discovered that BY-2 cells could be incubated with aphidicolin (a reversible inhibitor of replicative DNA polymerase α) and following removal of this drug, we could observe between 60 and 70% of cells reaching mitosis synchronously. Up to that time, published reports of cell synchrony in plants was no more than 10% (max). In fact we first established this system that could yield high levels of synchrony earlier in 1982. However, it was not necessarily reproduced by others, even in Japan! Some people seemed to doubt what we had done because they could not reproduce it. Professor Hiroh Shibaoka of Osaka University, who is now in retirement, knew me well and sent one of his younger staff

Dr. Tatsuo Kakimoto, to learn the technique and how to handle the BY-2 cells. However, after returning to Osaka, he called me 3–4 weeks later to say that he could not reproduce what he did in our laboratory. It took the Osaka group another 6 months to reproduce our results. The key reason why the technique could not be reproduced elsewhere was because new users of the cell line did not culture the cells under the precise conditions with which these cells must be grown: exactly 130 rpm at 27°C in darkness in specially modified Murashige and Skoog medium. I personally maintained the cell line for more than 27 years, but after retirement from the University of Tokyo, I stopped for one and a half years but recently reestablished it in my new laboratory. When I see the cells under microscopy, I can tell the condition of the cells.

After Shibaoka's group published their seminal work on the preparation of phragmoplasts (Asada et al. 1991) using highly synchronized tobacco BY-2 cells, our method was noted by workers particularly those outside of Japan. First, Professors Anne-Marie Lambert and Claude Gigot at the Institut de Biologique Moléculaire des Plantes du CNRS at Strasbourg were very enthusiastic about using our method and obtaining the cells from me. Given the lack of success of some laboratories with our line, I decided that it was absolutely necessary to describe full details of the correct way to handle and synchronize the cells. So, I wrote a review paper including technical details (Nagata et al. 1992). The paper has been cited more than 549 since the publication and the use of the cell line has spread to at least 35 countries world-wide; later, I decided to supplement this paper with another review (Nagata and Kumagai 1999). My hope was that the BY-2 cell line could be the plant HeLa cell line, a hope that has been fully realized.

4.2 Dedifferentiation

Dedifferentiation is a process, in which differentiated nondividing cells become meristematic. The molecular basis of dedifferentiation is poorly understood. I decided to study this process using tobacco mesophyll protoplasts. With this system, I could study cell division in protoplasts that could be induced to divide synchronously in a simple medium only supplemented with auxin and cytokinin (Nagata and Takebe 1970; see above). Thus, we followed auxin-induced genes during the first day of culture. These genes encode glutathione *S*-transferase (Takahashi et al. 1989; Takahashi and Nagata 1992), and are first expressed shortly after the addition of auxin, and their expression lasts up to 12 h. They are components in a mitogenic signaling process (Takahashi et al. 1995), although the precise function of GSTs in this process remains to be clarified. Among various types of GSTs (Marrs 1996), we discovered parA that is localized in the nucleus and is classified as a Type III GST, while parB that is localized in the cytoplasm belongs to a Type I GST. With a similar strategy, a cytokinin-induced gene was also identified (Iwahara et al. 1998), whose function also remains to be clarified.

Regarding this issue, we were always searching for plant hormone-regulated genes under the assumption that auxin and cytokinin are necessary for the induction of cell division. When I looked at what happens if auxin or cytokinin alone was given to the protoplasts, intriguing effects were observed. Regarding cell cycle progression, almost nothing happened with auxin alone, while the sole addition of cytokinin induced a small but significant level of DNA synthesis; however, this DNA synthesis did not go further and was not followed by cell division. The addition of auxin around 24 hr after the addition of cytokinin resulted in full cell cycle progression (our unpublished results). However, although the sole addition of cytokinin induced cells to undergo the transition from G_0 to S phase, cells did not progress into mitosis. It seems that during the early stage of dedifferentiation, there is a cytokinin-responsive stage shortly after the addition of cytokinin (G_0/S) and then, an auxin-sensitive stage (G_2/M) that follows. Currently, we are looking for what kind of molecular components of the cell cycle machinery are involved in this process.

4.3 Habituation

Habituation is an epigenetic change observed in cells in culture where after a prolonged continuous culture with a plant hormone like auxin, they develop the competence to grow on an IAA-depleted medium. It was discovered by Gautheret (1942), but its molecular basis has not been clarified although there have been many publications on this issue. Thus, habituation is a most enigmatic issue in plant cell and tissue culture. Regarding this, I have been culturing the tobacco 2B-13 cell line since 1988, which is derived from tobacco BY-2 cell line. The presence of auxin is obligatory for propagation of tobacco BY-2 cells, whilst depletion of auxin from the culture medium causes cell division to stop (Ishida et al. 1993). However, the 2B-13 cells can grow without auxin and thus, it shows a classic feature of habituation. After several trials to find differences between the two cell lines, it was found that addition of the culture filtrates from 2B-13 cells to auxin-starved nondividing BY-2 cells caused the induction of cell division. The search for factors that induced such cell division in this fraction revealed that they were glycoproteins, which when further purified by column chromatography and eluted with SDS-PAGE, were shown to be a single band of 30 kDa. It was finally identified by MALDI/TOF-MS analysis to be a kind of ATP-binding cassette (ABC) transporters named P-Glycoprotein (PGP) (Shimizu et al. 2006). However, it was also found that there are similar glycoproteins in the culture filtrates of tobacco BY-2 cells, but they had different molecular sizes of 25 and 40 kDa than those isolated from 2B-13 cells. Further characterization of these factors should give clues to understand the molecular basis of habituation. Regarding this issue, the molecular mechanism of auxin action has been well characterized and its signaling pathway has also

been described (Dharmasiri et al. 2005); however, its relevance relating to the above-mentioned factors remains to be clarified. This is one of my current interests at Hosei.

4.4 Heterophylly

I was interested in studying heterophylly that is observed in plants growing along the riverside that can tolerate submergence. This is because submergence causes morphological changes to the leaves. As certain environmental changes cause such distinct morphological changes, it is quite intriguing to follow the signaling pathway downstream from an environmental cue to morphological changes. This study began when Asuka Kuwabara joined us as a graduate student. We first established a system, in which induction of heterophylly in *Ludwigia arcuata* (Onagaraceae) can be conducted in sterile conditions and consequently, experiments can take place throughout the year, in contrast to the previous experiments in natural conditions, in which material sometimes deteriorates upon infection by microbes. Then, we found that ethylene induced elongated submerged-type leaves, while abscisic acid (ABA) induced round terrestrial-type leaves (Kuwabara et al. 2001, 2003). Most previous works on heterophylly had been explained by the elongation of leaf epidermal cells; these two plant hormones induced cell division but form two types of leaves of elongated and round shape leaves.

So again I encountered "cell division" during leaf development. Although cell divisions causes the increase in cell numbers in tissues and the increase of tissue mass, the plane of cell division axis to the long axis of leaves was different between ethylene and ABA treatments. This observation enabled us to analyze the processes involved in forming leaf shape mediated by cell division and as regulated by ethylene or ABA. This process can be divided into a few steps; first, how the direction of cell division plane would be determined by either a condition of submergence or terrestrial condition and then, how the respective cell division events contribute to affect leaf shape. These analyses revealed that there is a gradient in developmental stage in *L. arcuata* leaves; namely, leaf shape determination, starts from the tip to the base. Thus, at a certain stage of leaf development, differentiation in the tip is fixed, while at the base, it is still responsive to cues. When an intermediate stage of leaf is transferred from a terrestrial to a submergence habitat, or from submergence to terrestrial, leaf shape becomes spoon or spear head shaped, respectively (Kuwabara and Nagata 2006), which is a kind of developmental plasticity. These analyses have allowed us to first construct a mathematical model of how respective cell division events contribute to leaf shape and to ask what might be the molecular basis of this process. Since almost nothing is known about the *L. arcuata* genome, we are trying to understand these processes in *Arabidopsis thaliana* in collaboration with Dr. A. Kuwabara and Professor Andrew

Fleming, Sheffield University. As a kind of complementary evidence, I add that changes described above were caused at temperatures above 28°C, while below 21°C, cell elongation contributes to determine leaf shape (Sato et al. 2008). This study should give clues to understand the role of cell division in leaf shape determination.

5 Concluding Remarks

I am now trying to bring this chapter to an end. Normally, such a review would be from a retired perspective, looking back at one's career. However, as mentioned above, I now have further opportunities to continue my work. I would be happy if I could provide answers to questions on dedifferentiation, habituation, and heterophylly. All of these phenomena are found to be related to cell division. Throughout my career, I have first tried to study certain phenomena at the physiological level and then at the cellular level. Repeatedly I arrive at a road-sign that says "cell division." If one speaks of cell division at the present time, it is mostly related to publications on genes that regulate cell cycle progression but, as I explain here, there are many other phenomena in which cell division is involved particularly in relation to plant morphogenesis. So I would be extremely happy, if I could give some reasonable answers to exactly how cell division is linked to developmental processes. Finally, I thank people, who have worked with me in the past and those who are currently working with me in my new surroundings. Without their help I would not have been able to make progress in my scientific journey at any location or any situation. Thus far, I have enjoyed my journey in science but as such the journey never-ends; I hope I am only half-way.

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Genetics

Replication of Nuclear DNA

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Contents

Abstract In common with other eukaryotes, plant DNA is organised for replication as multiple replicons. A key event is the recognition of origins by the origin-recognition complex (ORC). Despite earlier indications, it is now likely that as in

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mammals, plant replication origins are not defined strictly by sequence; it is thus important to ascertain what features of origins are recognised by the ORC. Activation of origins occurs by stepwise loading of the pre-replication complex, all components of which have been identified in plants. This step is important in restricting DNA replication to once per cell cycle, although in plants, this restriction is relatively easily overcome, thus permitting DNA endoreduplication. It is also important to note the flexibility of origin use in relation to aspects of plant development. Is this flexibility mediated by ORC binding or at the pre-replication step? Following origin activation, the origin is prepared for initiation, again by the stepwise loading of several proteins, including the initiating DNA polymerase, polymerase-α-primase. The last pre-initiation step is strand separation by GINS-CDC45-MCM2-7 to generate a replication bubble. Synthesis itself is initiated by DNA polymerase-α-primase (which has been extensively researched in plants). This then "hands over" to DNA polymerase-ε on the leading strand and to DNA polymerase-δ on each Okazaki fragment. Polymerases -α, -δ and -ε are three of the 14 DNA polymerases known at protein and/or gene level in plants. Intriguingly, polymerase-β (a repair enzyme in animals) is associated in plants with DNA endoreduplication, in which replication is repeated without an intervening mitosis. Whether synthesised during replication or endoreduplication, the newly synthesised strands are completed by the ligation of Okazaki fragments and replicon-length pieces by DNA ligase 1. What will now be especially interesting is the integration of these molecular events into higher levels of control, such as the specification of meristem identity.

1 Introduction

The replication of DNA is the most fundamental molecular event undertaken by living organisms. This alone means that the process is of major interest to biologists. The chemistry of DNA does not of course vary from organism to organism: All must have evolved mechanisms that work with this essential chemistry to reach the same biochemical endpoint. However, there are differences between organisms that relate to the way that the DNA is organised and packaged in cells, to the very variable life-forms amongst living organisms and to their equally varied lifestyles. Each group is, therefore, worthy of attention in order that we can discern the universal features of DNA replication, the features that are common to, e.g. eukaryotes as opposed to prokaryotes and the features that are specific to particular groups. The latter are likely to be related in turn to the control of DNA replication in context of the regulation of cell division within the body and the life of the organism.

In this chapter, DNA replication at the molecular level has been focussed on, using wherever possible plants as the example, but sometimes needing to refer to other eukaryotic organisms. As with many other areas of molecular and biochemical research, work on plants had lagged behind that on vertebrate animals and on model unicellular eukaryotes, such as budding yeast or fission yeast. However, the

research climate is changing and, as will be apparent from the text, we are beginning to see real progress in this important area.

2 Organisation of DNA as Units of Replication

All eukaryotic organisms organise their DNA for replication as multiple units known as replicons. In cells undergoing DNA replication, these multiple units are readily visualised by fibre autoradiography (Cairns 1966; Huberman and Riggs 1968). This was first applied to plants by Van't Hof (1975) and has been widely used in studies of plant DNA replication. In more recent versions of the technique, radioactive label has been replaced by fluorescent label (e.g. Quélo and Verbelen 2004) and new methods for preparing the DNA for microscopy, such as DNA combing, have been developed, giving overall a better resolution of the replicating molecules (reviewed by Bryant and Francis 2008). Nevertheless, the general picture remains the same. A replicon is defined as a tract of DNA replicated from one origin of replication (*ori*). Replication proceeds outwards in two directions from the origin to the termini (as visualised by "step-down" fibre autoradiography: See Bryant et al. 2001). It is still not clear whether the termini are specific sites or simply zones, in which replication forks finally stall. There is some evidence that specific termini exist in the replicons within the repeated genes that encode rRNA in pea (Hernández et al. 1988b), but this may not be the general case. For example, under conditions in which fewer replication origins than usual are utilised, replication continues through sites/zones, which would in other circumstances be or contain termini.

Measurement of "fork rates", i.e. the rates at which the labelled tracts of DNA increase in length as the labelling times are extended, shows that the replication of any individual replicon takes much less time than is taken by the whole S-phase. This is typified by pea (*Pisum sativum*), in which an individual replicon is replicated in ca. 2 h, but in which S-phase is four times longer (Van't Hof and Bjerknes 1981). It was in response to similar data in mammals that the concept of replicon families was developed (Jasny and Tamm 1979), suggesting that replicons are organised as time-groups or families. The concept embodied the idea that each family has its own particular time within S-phase, during which it is active in replication. Thus in the small genome of *Arabidopsis thaliana*, which has ca. 30,000 replicons, there are only two families, of which one family completes replication in the first 2 h of S-phase. Initiation of replication in the second family is delayed until 35–40 min after the start of S-phase; this family also takes about 2 h to complete replication (Van't Hof et al. 1978). The two families "fire" in the same order in all S-phases, thus implying a specific temporal control to ensure that replicon families are replicated in a specific order. However, the general application of this model has been challenged. It may well be true for plants and possibly for budding yeast, but in vertebrates the situation is a more complex one, in which some replicons may have a specific "time-slot" in S-phase, but others do not (Paixao et al. 2004; Jeon et al. 2005; see also the reviews by DePamphilis 2003; Bryant and Francis 2008). The same situation exists in fission yeast (Patel et al. 2006).

3 Characterisation of Replication Origins

When replicating DNA is visualised by fibre autoradiography or fluorescence labelling, it is possible to measure origin-to-origin distances (i.e. replicon lengths) on any individual DNA strand (see Bryant et al. 2001). It is often observed that there is a clear mode of replicon length with some variation on either side of the mode and that this modal length varies between species. Thus, Van't Hof and Bjerknes (1977) obtained a modal replicon size of ca. 54 kb in pea root meristems, while Francis and Bennett (1982) found a replicon length of 60 kb in rye (*Secale cereale*). However, there is also extensive evidence that replicon size may be flexible. Thus, in several plant and animal species, there are well-defined situations in which the replicon size shortens significantly, implying that more origins must be employed. There are also, as mentioned briefly above, situations in which replicon length is increased. These phenomena are discussed in more detail later in Sect. 4.

Although replication origins may be "seen" in fibre autoradiographs or in fluorescence micrographs, it is much more difficult to separate a functional origin from the rest of the DNA. In unicellular eukaryotes, such as budding yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*), it has proved possible to identify origin sequences by their ability to confer autonomous replicative ability on plasmids, which lack their own replication origin. In *S. cerevisiae*, these *ars* elements have been shown by 2D gel electrophoresis to be bona fide origins of replication (Brewer and Fangman 1987; Huberman et al. 1988). In the same yeast, the *ars* elements contain a conserved 11 bp sequence that is essential for their function (Maharhens and Stillman 1992): $^{A}/_{T}$TTTTAT-$^{G}/_{A}$TTT$^{A}/_{T}$. However, there is not a similar tight sequence requirement in *S. pombe* (see reviews by Bryant et al. 2001; Bell 2002; Bryant and Francis 2008).

The yeast *ars* plasmid assay (Stinchcomb et al. 1980) has been used to analyse the DNA of several multi-cellular eukaryotes, including some plants (e.g. Sibson et al. 1988). All these organisms have invariably been shown to possess the *S. cerevisiae* essential *ars* sequence. However, whether the sequence acts as or is part of a replication origin in any multi-cellular eukaryote is far from clear.

Direct isolation of plant DNA replication origins has proved very difficult. However, multi-cellular eukaryotes possess an origin of DNA replication in each non-transcribed spacer (NTS) between the repeated genes that code for rRNA. The "replication bubbles" associated with initiation at these sites are visible in electron micrographs of replicating DNA in *Drosophila*, *Tetrahymena* and *Xenopus* (reviewed by Van't Hof 1988). Van't Hof's group (Van't Hof et al. 1987a, b; Hernández et al. 1988a) have taken advantage of the high level of repetition of these genes in pea: In cultured pea root meristems, they localised the site of initiation of replication to a 1,500 bp region within the NTS. Fractionation of replicating DNA by 2D gel electrophoresis confirmed that the replication bubble that marks the initiation of strand separation occurs within this region (Van't Hof and Lamm 1992). Sequencing shows that this region contains a very AT-rich domain that includes four good matches to the *S. cerevisiae ars* core (Hernández et al. 1988a, 1993).

On this basis, it appears that the budding yeast *ars* core sequence may also be part of plant origins, despite the absence of this sequence in *S. pombe* origins (as discussed above). However, to date, there has been no direct demonstration of this. Further, since no other plant nuclear DNA replication origin has been isolated, it is not known whether the *ars* core sequence is a universal feature of plant origins. One possible line of evidence to suggest that it may be is the finding that replicating DNA isolated in very early S-phase in synchronised pea root meristems is enriched for AT-rich DNA, including the *ars* core sequence or close matches thereto (Bryant 1994). However, it was not demonstrated that these sequences actually contained any replication origins.

Clearly, there has been little recent progress in the direct characterisation of plant replication origins. We thus turn to consider the replication origins of other organisms, noting that a DNA replication origin database has now been established (Nieduszynski et al. 2007; see also the review by Bryant and Francis 2008). There are some rare examples of origins in animals, in which a specific sequence may be involved. For example, there is an *ars* core sequence at or very close to the preferred initiation site in the mammalian rDNA NTS (Coffman et al. 2005), as has already been described for the equivalent site in plants. The origins of replication from which the chorion genes in *Drosophila* are amplified are also AT-rich and contain sequences very similar to the *ars* core (Austin et al. 1999; Spradling 1999). However, specific sequences in origins of replication are the exception rather than the rule. Thus, some investigators have suggested that initiation of replication in higher eukaryotes has much "looser" sequence requirements than in budding yeast (DePamphilis 1993; Bogan et al. 2000; Bell 2002; Antequera 2004; Schwob 2004; Cvetic and Walter 2005). This lack of specific sequence is seen in plasmid-based assays for initiation of replication in mammalian cells, in the analyses of origins detected by DNA combing and in micro-array-based genome-wide analyses. Indeed, with over 20 different mammalian replication origins now characterised, we cannot distinguish any common sequence that is shared by them all. Just as in *S. pombe*, the overall general feature of these origins is that they are A–T-rich and some contain tracts of bent or curved DNA. Both these features facilitate strand separation (see Marilley et al. 2007 for a discussion of DNA denaturation in relation to DNA curvature in fission yeast). Another frequent, but not universal, feature of mammalian origins is that the A–T-rich regions are located in the vicinity of CpG islands (Paixao et al. 2004; reviewed by Antequera 2004 and Schwob 2004). The specific tract of DNA that acts as an origin may be closely defined over a few hundred base pairs, as in the origin associated with the mammalian lamin-B2 gene (Paixao et al. 2004) or may be spread over several kilobases (Dijkwel et al. 2002). In the latter type of origin, there may be several possible initiation sites any one of which may be used in any one cell cycle (DePamphilis 2003).

Further, at the extreme end of "loose" sequence requirements, there are instances in animal cells in which initiation can take place completely regardless of sequence (Mechali and Kearsey 1984; Gillespie and Blow 2000). These situations are admittedly somewhat specialised, such as the very rapid phase of DNA replication and cell division in early embryogenesis in amphibia (Gillespie and Blow 2000).

Here, active origins are very closely spaced and indeed, the spacing may be determined by the amount of "room" available to accommodate the protein complexes involved in initiation. Later in embryogenesis, from the mid-blastula transition onwards, when cell division is much slower, more "normal" spacing is seen. These data thus suggest that when DNA replication needs to be rapid, any requirement for general features of sequence or chromatin structure can be abandoned. However, it is emphasised that we do not know whether this happens in plants.

4 Flexibility of Origin Use

Study of DNA replication during different developmental phases in plants shows that origin-to-origin spacing and thus, replicon length may vary. This variation may occur in relation to different phases of development, in response to nutrients or hormones or as response to experimental manipulation. For example, in *Sinapis alba*, the floral stimulus induces a halving of the modal replicon length from 15 to 7.5 kb in the shoot meristem, i.e. twice as many origins are utilised. This contributes to the dramatic shortening of the S-phase (Jacqmard and Houssa 1988). The same feature is seen during the transition to flowering in *Silene coeli-rosa* and *Pharbitis nil* (Durdan et al. 1998). In *Sinapis*, this aspect of the floral response may be mimicked by application of the hormone cytokinin (Houssa et al. 1990). This effect of cytokinin is not confined to the shoot apex. Recruitment of extra origins is also seen when the hormone is applied to dividing cells in the vegetative shoot apex of a grass, *Lolium temulentum* and in the ovule of tomato (*Lycopersicon esculentum*) (Houssa et al. 1994). The most dramatic example in plants of recruitment of extra origins was seen when the DNA of synchronised pea root meristems was cross-linked with psoralen in order to stall movement of the replication forks. This caused extra origins to be recruited between the cross-links, dramatically shortening the origin-to-origin distance (Francis et al. 1985). All these data suggest that plant DNA contains sequences that normally do not act as origins, but can be called into action under particular circumstances.

However, the reverse is also true: Origins that are normally in use can be inactivated. An example of this is seen when addition of trigonelline to lettuce roots causes a halving of the number of active origins, with two out of every cluster of four being silenced (Mazzuca et al. 2000). In the *S. alba* shoot meristem, abscisic acid caused a halving of the number of active origins and thus, a doubling of replicon length (15–30 kb: Jacqmard et al. 1995) (as opposed to cytokinin which causes "extra" origins to be activated, as described above).

An earlier suggestion that is consistent with all these data was that eukaryotic DNA contains "strong" and "weak" origins (Francis et al. 1985). Observations of initiation of replication in *S. pombe* are consistent with this idea: Clustered origins of replication demonstrate hierarchies of initiation frequencies (Dubey et al. 1994; Okuno et al. 1997; Kim and Huberman 1999). However, data from rye-wheat hybrids suggest further levels of complexity. Diploid rye (*S. cereale*) ($2n = 2x = 14$)

exhibits, as already noted, a modal replicon length of 60 kb (Francis and Bennett 1982), while in bread wheat ($2n = 6x = 42$) the modal length is 16.5 kb (Kidd et al. 1992) However, the allohexaploid hybrid triticale ($2n$ rye $\times$ $4n$ wheat) exhibits a completely "novel" modal replicon length of 15 kb (Kidd et al. 1992). The origin spacing in both genomes is thus re-set in the hybrid, a phenomenon ascribed to "unknown factors within the triticale nucleoplasm" (Bryant and Francis 2008). Although these data may still be consistent with the idea of strong and weak origins, it is much harder to reconcile them with the view that initiation always occurs at specific sequences. Indeed, the question of which features of sequence and/or chromatin structure contribute to "strength" or "weakness" of origins remains completely unanswered.

Further, based on data from yeasts and metazoa, features of chromatin related to transcription also have an effect on origin distribution and/or usage (reviewed by Bryant and Francis 2008). In both budding and fission yeast, many origins are located in intergene regions (Dai et al. 2005). In mammals, proximity to promoters is a common (but not universal) feature of origin position. Origins in transcriptionally active regions of chromatin tend to be active earlier in S-phase than origins in less active regions (Gomez and Brockdorf 2004).There is also a correlation between hierarchies of origin activation and transcription patterns in budding yeast (Donato et al. 2006). Associated with this is the finding that regions of chromatin in which genes are rather sparsely distributed also contain few origins.

These relationships between transcription and origin activity lead to the question as to whether chromatin modifications related to transcription also affect origin activity (Zou et al. 2005) and there is indeed some evidence that this is so. Histone acetylation, a feature of transcriptionally active chromatin, is associated with origin activity in mammalian cells (Vogelauer et al. 2002), although it does not affect the ordered sequence of origin activity in budding yeast (Aparicio et al. 2004). In contrast to histone acetylation, DNA methylation reduces transcriptional activity and in Xenopus, also appears to block the activation of origins (Harvey and Newport 2003).

Major changes in transcriptional activity are of course part of the process of cell and tissue differentiation. The linkage between transcription and origin activity in metazoa (but not yet demonstrated in plants) then leads to the suggestion that differentiation may involve dramatic changes in patterns of origin usage. It is possible that the change in origin spacing observed during the floral transition (discussed earlier) represents such a change (although equally it may simply reflect the recruitment of more origins within the same overall pattern). In mammals, however, it is now very clear that differentiation leads to a change in the pattern of origin use. This was first demonstrated in the development of B-cells (Norio et al. 2005) and has now also been reported during the differentiation of mouse embryonic stem (ES) cells (Hiratani et al. 2008). In their excellent paper, the latter authors report the construction of "high-resolution replication-timing profiles" showing that differentiation from ES cells to neural precursor cells is accompanied by a "global reorganisation of replication domains". Whether this type of phenomenon also occurs in plants, which have less rigid epigenetic controls than mammals, remains to be seen.

5 Biochemistry of Origin Usage

5.1 Introduction

It is self-evident that origins of replication are the places at which DNA replication starts. Four phases are recognised in this overall process: Origins are first recognised, activated, a pre-initiation complex is formed and finally replication itself is initiated by the laying down of the first nucleotide. These processes involve an array of proteins that is much more extensive than was thought probable 20 years ago. Our knowledge of them has largely been built up from work with budding yeast and fission yeast and with metazoa. It is clear that DNA replication starts in essentially the same way in all these eukaryotes, although there are some minor variations in the complement of proteins involved. There are also, as might be expected from the variety of "lifestyles" amongst these organisms, more significant variations in aspects of overall regulation. Work with plants has sadly featured very little in building up the overall picture (Bryant et al. 2001). However, in an excellent recent paper (Shultz et al. 2007) it has been shown, based on genome-wide analyses, that plants do indeed possess the biochemical "machinery" that conforms to the general pattern.

5.2 Recognition of Origins

It has already been noted that in budding yeast, replication origins contain a specific DNA sequence, the *ars* core sequence. This led to the identification of proteins that bind specifically to the *ars* and thus, to the discovery of the origin-recognition complex (ORC) (Bell and Stillman 1992). Many investigators, including the author of this chapter, had been searching for such proteins and it was the finding that binding to the *ars* core sequence requires ATP that led to the breakthrough. This discovery was thus a true turning point in our understanding of the initiation of DNA replication. In budding yeast, the ORC is a complex of six proteins, five of which determine the sequence requirements (Lee and Bell 1997). The ORC in effect "marks" origins of replication and in budding yeast, the complex is bound to the origin for nearly the whole of the cell cycle (Diffley and Cocker 1992; Dutta and Bell 1997). The ORC plays no direct role in origin activation or in initiation. Nevertheless, the strict sequence requirements for ORC binding in budding yeast directly parallel the strict sequence requirements for origin function, showing that this marking of the origins by the ORC is essential for the initiation of DNA replication.

Subsequently, ORC proteins have been identified by direct biochemical characterisation and by proteomic and genomic analyses in a range of other eukaryotes. Most of the organisms studied, including plants (Gavin et al. 1995; Witmer et al. 2003; Collinge et al. 2004; Mori et al. 2005b; Shultz et al. 2007) and mammals

(Dhar et al. 2001) possess six ORC proteins. Identification of ORC proteins in other eukaryotes also throws light on the sequence requirements for initiation of replication. At one end of the range, a complete lack of sequence specificity is seen in the replication of sperm DNA in a cell-free extract of *Xenopus* oocytes (Gillespie and Blow, 2000). In this somewhat unusual system, replication origins occur at 10 kb intervals and it is indeed the binding of the ORC, irrespective of sequence, that sets this up. At the other end of the range, in the amplification of the chorion genes in *Drosophila*, the ORC binds to a specific site within a defined 440 bp tract of A–T-rich DNA (Austin et al. 1999).

However, both these situations are exceptions to the general rule. In organisms ranging from fission yeast to vertebrates (and including cell-cycle related, rather than amplification-related, DNA replication in *Drosophila*), direct study of DNA–protein interactions as well as use of DNA "footprinting" and ChIP techniques have mostly shown a general requirement for AT-richness and in some instances for strand asymmetry (Antequera 2004; Keller et al. 2002; Ladenburger et al. 2002; Schaarschmidt et al. 2004). In fission yeast, the binding of the ORC to the AT-rich origins (and especially to the oligo-A tracts therein) is mediated by protein domains known as AT-hooks (Chuang and Kelly 1999). We currently have no information on sequence recognition by plant ORC proteins, but there is no reason to suppose that they differ in sequence requirements from the general pattern presented here.

5.3 Activation of Origins

As with other aspects of DNA replication, data from budding yeast and from fission yeast are very helpful in setting the scene. In budding yeast, prior to the activation phase, one more protein binds to the ORC at the origin. This protein, NOC3, was first discovered as a nucleolar organiser protein, but here appears to be involved in preparing the marked origin for activation (Zhang et al. 2002). The *NOC3* gene is present in both the Arabidopsis and rice genomes (Shultz et al. 2007), but there is no direct evidence of a role in pre-activation at the origin. Activation itself involves the successive recruitment of several more proteins at the origin as revealed initially by an increase in the length of the tract of protected DNA (as shown by DNAse protection footprinting experiments) from late anaphase and throughout the G1 phase (Diffley et al. 1994; Cocker et al. 1996). In budding yeast, this can only happen at origins that have been "marked" by the ORC (Cocker et al. 1996; Kearsey et al. 2000) and it is presumed that this is also true in other eukaryotes. However, as already noted, the actual initiation site may vary within a tract of DNA that acts as an origin. This flexibility is at least partly due to the behaviour of some of the proteins involved in activation, as is now discussed.

The first of the proteins recruited to the origin in the activation or pre-initiation phase is CDC6 (cdc18 in fission yeast) (Cocker et al. 1996). This in turn recruits a complex consisting of CDT1 and MCMs2-7 (Nishitani et al. 2000; see also reviews by Bryant and Francis 2008 and Shultz et al. 2007). The MCMs were first

discovered in budding yeast as proteins essential for mini-chromosome maintenance. The next stage of activation is the hydrolysis of ATP mediated by the ORC and CDC6 acting together (Randell et al. 2006. This allows the release of CDT1 from the origin (although in some models, CDT1 may remain until the next phase: see Bryant and Francis 2008) and also facilitates the binding of the MCM2-7 complex (which is ring-shaped) around the DNA (Waga and Zembutsu 2006). This array of proteins (the ORC, CDC6 and MCM2-7) is known as the *pre-replicative complex*.

Since MCM2-7 act as the helicase that separates the two DNA strands and then moves away from the initiation site generating the two replication forks (Labib et al. 2000), it might be expected that two copies of the MCMs are loaded at this activation stage. However, the situation is more complicated than this. It was noted by Blow and Dutta (2005) that MCM2-7 proteins are much more abundant than the ORC or CDC6. Further, the ORC–CDC6-mediated hydrolysis of ATP may be repeated, allowing several MCM2-7 complexes to bind around the DNA in the vicinity of one ORC (Woodward et al. 2006) In terms of helicase activity, this appears to be excessive. However, it does make sense of the observation that, in some eukaryotes, the site of initiation is not specifically fixed in relation to the short tract of DNA marked by the ORC (DePamphilis 2003). In this way, one ORC can make available a long tract, even extending to several kb, in which initiation may occur at one of several "convenient" sites that are presumably AT-rich (and possibly curved: Marilley et al. 2007) and therefore, more prone to denaturation (Dimitrova et al. 2002).

The proteins involved in activation of origins, namely CDC6, CDT1 and MCMs2-7 have all been detected in plants, most of them at all levels from the gene to the protein and including the mRNA (Dambrauskas et al. 2003; de Jager et al. 2001; Dresselhaus et al. 2006; Lin et al. 1999; Masuda et al. 2004; Moore et al. 1998; Ramos et al. 2001; Sabelli et al. 1996; Shultz et al. 2007). Interestingly, one of the plant MCM2-7 complex was initially discovered through characterisation of a mutant. Mutants, such as the temperature-sensitive cell cycle mutants of both budding and fission yeast, are rather less useful in diploid organisms. However, this problem may, in some instances, be overcome by gene trap transposon mutagenesis. This technique was used to identify the *PROLIFERA* gene in Arabidopsis, initially identified as an *MCM* gene (Springer et al. 1995) and subsequently shown to encode MCM7 (Springer et al. 2000).

Complementation genetics has also proved its worth. In this approach, genes or cDNAs are introduced into budding yeast or fission yeast mutants to establish whether the plant sequence will complement a mutation in the yeast. Thus, the identity of the gene encoding MCM2 in rice was confirmed by its ability to complement MCM2 (cdc19) mutants in fission yeast (Cho et al. 2008).

In "normal" cell cycles, activation of origins occurs only once in each cycle and thus, the origins rapidly revert to an inactivated state in which initiation of DNA replication can no longer occur. The transition from the inactive to the activated state is often referred to as "licensing" of the origin for replication: The cell is only "licensed" or "permitted" to replicate its DNA when origins are

activated. Licensing is regulated at both cellular and molecular levels. The former, mainly based on the activities of cyclins and cyclin-dependent kinases, lies outside the scope of this review. At the molecular level, as mentioned in later sections, the dissociation of the CDC6 protein from the pre-replicative complex (in fission yeast this is driven by a cdc2-kinase-mediated phosphorylation: Lopez-Girona et al. 1998) and its subsequent degradation (Drury et al. 1997) are key elements in the once-per-cycle licensing. Equally important is the transcriptional regulation of CDC6, exemplified by data from synchronised tobacco BY2 cells (Dambrauskas et al. 2003) and from Arabidopsis (de Jager et al. 2001). In both, maximal expression of CDC6 occurs in early S-phase and then declines to almost undetectable levels by the end of G2. Expression of the MCMs by contrast occurs throughout the cell cycle (Dambrauskas et al. 2003) and the proteins and/or mRNAs are readily detectable in dividing cells throughout the body of the plant (Cho et al. 2008; Holding and Springer 2002; Huang et al. 2003; Springer et al. 2000). In common with other proteins active in S-phase, the promoters of the *CDC6* and *MCM3* genes, both possess the E2F transcription factor binding site (two copies in the *MCM3* gene) (Dambrauskas et al. 2003; Stevens et al. 2002) despite their different patterns of transcriptional regulation.

5.4 The "Pre-Initiation" Phase: Getting Ready to Replicate

The template is now in a state where the final preparations for synthesis are made. Some authors (e.g. Shultz et al. 2007) call this phase "initiation" but in strict biochemical terms, initiation is the laying down of the first nucleotide in the new DNA strand. Hence the term, "pre-initiation" is used here. The number of proteins involved in this phase is remarkable and it needs to be said that for some of them we do not as yet have any clear idea of their role or indeed of the order in which they participate. The order in which the events are presented here is currently a best fit to the data.

At the end of the previous phase, the origin and the adjacent tracts of DNA were loaded with one ORC, with one CDC6 and with several copies of the MCM2-7 complex. Now, CDC6 is displaced from the origin, a step which is necessary for the final activation of MCM2-7. Given the number of MCM2-7 complexes present in the region of a single origin, in most instances, there must be some selection of those MCM complexes that are activated. Nothing is known about the mechanism of selection. Further, there is the possibility that, under particular conditions, including "replicative stress" more MCM complexes may be activated during S-phase (Woodward et al. 2006). This would be compatible with the findings made by Francis et al. (1985b): In pea root meristems, cross-linking the DNA to prevent movement of replication forks led to the establishment of more initiation sites.

Activation of MCM2-7 starts when the ORC and CDC6 are phosphorylated by CDC7-DBF4 and CDC28 (cdc2)-CLN1 (Jackson et al. 1993). This leads to the

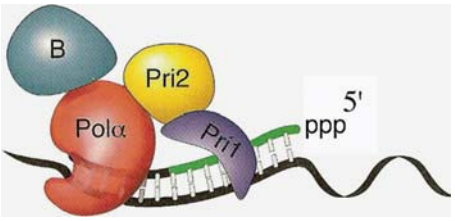

Fig. 1 The DNA polymerase-α-primase complex. Reproduced, with permission, from Frick and Richardson (2001)

displacement of CDC6 and its destabilisation. The next step is the joining of another MCM, MCM10 to the complex. This protein appears to play several roles from this point on and its presence in the pre-initiation complex is essential for successful DNA replication, at least in budding and fission yeast (Homesley et al. 2000; Moore and Aves 2008). Its first function is to stimulate the phosphorylation of MCM2-7 by CDC7-DBF4. MCMs2-7, acting with MCM10 then recruit the DNA-polymerase-α loading factor CDC45. Association of TOPBP1 with CDC45 is essential for this step. Indeed, the ability if TOPBP1 to bind with CDC45 is much greater in late G1/early S than at any other stage of the cell cycle (Schmidt et al. 2008). The mechanism for loading the polymerase-α-primase onto the template is incompletely understood. In one model, CDC45 binds directly to the p70[1] (non-catalytic) sub-unit of DNA polymerase-α, which then recruits the two primase sub-units and the p180 (catalytic) sub-unit of the polymerase (Arezi and Kuchta 2000). In a second model (Uchiyama and Wang 2004), it is proposed that the non-catalytic sub-unit of DNA-polymerase-α, p70, remains anchored to one of the sub-units of the ORC, ORP2 and then, after CDC45 binds to the origin, recruits firstly the p58 sub-unit of primase and then the remaining two sub-units, one of primase and one of polymerase-α, to complete the complex (see Fig. 1). The third model envisages that although the loading of the polymerase-α-primase is dependent on CDC45, it is actually MCM10 that interacts directly with the polymerase-α-primase complex to bring it to the template (Ricke and Bielinsky 2004).

The four proteins of the GINS complex (PSF1-3 and SLD5) (see Shultz et al. 2007), plus MCMs 8 and 9 are also loaded into the pre-initiation complex at this stage (Gozuacik et al. 2003; Maiorano et al. 2005; Yoshida 2005). Finally, the helicase activity of the MCM2-7, in concert with CDC45 and the GINS proteins function as a helicase (Pacek et al. 2006) to separate the two DNA strands and form a replication "bubble", which may be up to several hundred base pairs in overall length (see Bryant 2008). As replication gets under way (see below), the bubble is extended by the continued action of the MCM2-7-CDC45-GINS helicase to establish the two replication forks. Whether or not any other helicase is also involved in

[1]The size of this polypeptide varies between 70 and 90 kDa in different organisms.

Table 1 Summary of the pre-initiation events – getting ready to replicate at an activated origin

Order of events	Events
0	Origin is already loaded with ORC, CDC6, MCM2-7
1	ORC and CDC6 phosphorylated by CDC7-DBF4 and CDC28-CLN1
2	CDC6 displaced from origin and de-stabilised
3	MCM10 recruited to origin
4	MCM10 stimulates phosphorylation of MCM2-7 by CDC7-DBF4
5	CDC45 associates with TOPBP1 (essential for event 6)
6	MCM2-7 and MCM10 recruit CDC45 to the origin

fork movement is not clear and indeed the situation may vary between eukaryotes (Bryant 2008). The single-stranded state is stabilised by the trimeric single-stranded DNA binding protein, RPA. Pre-initiation is thus a complicated stage in the overall process, and to aid understanding, it is summarised in Table 1. It may be noted in passing that the DNA template is now very crowded, especially because it is still complexed with nucleosome core particles. The latter are only displaced in front of the moving replication forks (formation of the initial replication bubble initiates this) and are re-assembled behind the forks. The level of phosphorylation of the linker histone H1, is important here (Thiriet and Hayes 2009). In *Physarum polycephalum,* phosphorylation leads to the transient loss of H1 from chromatin; nucleosome displacement can then occur. Thus, the timing of replication in a particular chromatin domain may be at least partly determined by the level of H1 phosphorylation in that domain (for other details of chromatin re-modelling during replication, see Kohn et al. 2008).

Genes encoding all these proteins are known from Arabidopsis and rice and, in some instances, from several other species too (reviewed by Shultz et al. 2007). We do not have detailed information of the protein–protein interactions nor of the specific steps involved in building the pre-initiation complex in plants, but given the conservation of the essential coding sequences, there is no reason to suppose that these processes in plants differ significantly from what is described here. Some data are available concerning regulation of CDC45 in Arabidopsis: The transcript is detected in populations of dividing cells and a peak of expression occurs at the G1–S boundary (Stevens et al. 2004).

Garcia-Maya and Buck (1997) were the first to isolate and purify a plant (tobacco) RPA (replication protein A), clearly identifying the three protein subunits with molecular weights of 70 (RPA1), 34 (RPA2) and 14 kDa (RPA 3). The protein had a very high affinity for ssDNA and stimulated the activity of DNA polymerase-α fourfold (presumably because of maintenance of the single-stranded state of the template). RPA has also been extensively characterised in rice and to a lesser extent in Arabidopsis (Ishibashi et al. 2005, 2006). In these species, the three protein subunits have molecular weights of 70 (RPA1), 32 (RPA2) and 14 kDa (RPA3). In Arabidopsis, there are five separate *RPA1* genes, two *RPA2* genes and two *RPA3* genes (Shultz et al. 2007). The numbers of these genes in rice are three, three and one respectively (Ishibashi et al. 2006). Characterisation of RPA at the protein level in rice has revealed three different RPA trimers (1a-2b-3, known as RPA-A; 1b-2a-3,

known as RPA-B; 1c-2c-3, known as RPA-C) (Ishibashi et al. 2006). Studies of sub-cellular localisation place RPA-A in the chloroplast and RPA-B and C in the nucleus (Ishibashi et al. 2006). At sub-unit level, transcripts of RPA1a are present in a wider range of cells than those of RPA1b which are confined to dividing cells. In Arabidopsis, however, inactivation of RPA1a (part of RPA-A in rice) by insertional mutagenesis or by RNAi is lethal, suggesting that it has an essential function, most likely in nuclear DNA replication (Ishibashi et al. 2005). Inactivation of RPA1b (part of RPA-B) has no effect on phenotype, except that the plants are more sensitive to DNA-damaging agents, suggesting a role in repair. Finally, in a study that did not distinguish between protein isoforms, RPA2 protein abundance was shown to be cell-cycle regulated in rice (Marwedel et al. 2003), unlike the mammalian RPA2, which is largely regulated by post-translational phosphorylation.

6 The Replication Phase

6.1 Initiation

The initiating DNA polymerase, polymerase-α-primase is already loaded onto the template (see above). This enzyme is a tetrameric protein that contains two catalytic subunits, namely a polymerase and a primase (Fig. 1). None of the other polymerases involved in nuclear DNA replication possess primase activity and thus, only polymerase-α can initiate DNA synthesis de novo. Working first on the leading strand, this tetrameric complex lays down a mixed RNA–DNA primer: ca 11 ribonucleotides are inserted by the primase catalytic subunit followed by the polymerase catalytic subunit inserting a variable number of deoxyribonucleotides. The polymerase-α-primase complex then switches strands and on the lagging strand, initiates each Okazaki fragment in same way. In eukaryotes, Okazaki fragments are of the order of 200 bases in length and I have speculated previously that this is because the initiation of each one requires the displacement of a nucleosome core particle as the replication forks move outwards from the initiation site (Bryant and Dunham 1988).[2]

6.2 Extending the Primers

On the lagging strand, polymerase-α is displaced by DNA polymerase-δ, which completes the synthesis of each Okazaki fragment (Garg and Burgers 2005). This polymerase requires a processivity factor, PCNA (proliferating cell nuclear

[2] Sanchez et al. (2008) discuss the replication of the non-DNA elements of chromatin and the conservation of epigenetic marks during plant DNA replication.

antigen) to complete the task. PCNA is a ring-shaped trimeric protein, which opens up to encircles the DNA template and then, acts as a sliding clamp for the polymerase. The loading of PCNA onto the template requires another multimeric protein, RFC.

On the leading strand, the primer is extended by the third nuclear replicative DNA polymerase, polymerase-ε (McElhinny et al. 2008; Pursell et al. 2007) which, like polymerases-α and -δ, is a tetrameric protein. By virtue of its role on the leading strand, this is the most processive of the DNA polymerases, presumably able to synthesis several kilobases of daughter-strand DNA. It is, therefore, likely to require a processivity factor, but at present it is not clear what that factor is. It was initially described as a PCNA-independent enzyme, but it has subsequently been reported that its activity is increased by PCNA (Chilkova et al. 2007; Kelman et al. 1999; Maga et al. 1999). However, that stimulation may at least partly be ascribed to PCNA acting as primer-recognition protein. Other authors have suggested that the GINS complex is the processivity factor for polymerase-ε (Shultz et al. 2007), but it remains a possibility that once loaded onto the DNA at the primer terminus, it is the enzyme's high affinity for DNA that ensures its processivity.

6.3 Primer Recognition

Mention of the possibility that PCNA may act as a primer-recognition protein for polymerase-ε introduces a brief discussion of this topic. Primer-recognition proteins associated with DNA polymerase-α-primase have been reported in mammalian cells (Pritchard et al. 1983; Vishwanatha et al. 1992) and plants (Anderson et al. 2004: Bryant et al. 2000; Burton et al. 1997). In all these organisms, the primer-recognition protein increases the efficiency of use by DNA polymerase-α of primed templates, thereby stimulating DNA synthesis.

For both animals and plants, the situation is somewhat strange. Jindal and Vishwanatha (1990) showed that the mammalian primer-recognition protein is a heterodimer consisting of 36 and 42 kDa proteins. Characterisation of the two proteins showed that the former is identical to annexin II and the latter to the glycolytic enzyme, phosphoglycerate kinase (PGK). In other words, both proteins have other well-characterised roles in addition to acting as primer-recognition proteins. These proteins were thus amongst the first examples of "moonlighting proteins" (Jeffery 1999) to be described in mammals. It has also been inferred, but not directly demonstrated that the plant primer-recognition protein acts as dimer, in this instance a homodimer, consisting of two copies of a 42 kDa protein. All the evidence (summed up by Bryant 2008) points to the protein as again being identical to PGK; indeed, PGK can replace the primer-recognition protein in stimulating DNA polymerase-α activity on infrequently primed templates (Bryant 2008).

However, the question remains as to why polymerase-α should need a primer-recognition protein. It is understandable that polymerase-ε working on the leading strand may need assistance in locating primers, but polymerase-α is tightly

associated with its own primase activity. Furthermore, the evidence (Frick and Richardson 2001) suggests that the complex does not dissociate from the template between the primase laying down the RNA part of the primer and the polymerase extending it as a short tract of DNA. As discussed more fully by Bryant (2008), the situation is somewhat puzzling.

6.4 MCMs 8–10

In addition to the enzymes and other proteins at or around the replication fork, mentioned immediately above, MCMs 8, 9 and 10, loaded at the initiation site in the pre-replicative phases, are still present. For MCM 10, there is good evidence from fission yeast that it binds to DNA polymerase-α-primase and to DNA, thereby stabilising the polymerase catalytic subunit (see Moore and Aves 2008). For MCM8, there are the possibilities that it aids the binding of RPA and/or DNA polymerase-α-primase at the replication fork or that it "assists" the helicase activity of MCM2-7-CDC45-GINS, but further work is needed to clarify its role. For MCM 9, we currently have no information.

6.5 Maturation: Primer Removal

Removal of the primers occurs as shown in Fig. 2. The incoming polymerase displaces the RNA part of the primer and at least a section of the DNA part of the primer, creating a flap. This is removed by flap endonuclease, FEN1 and the polymerase fills any remaining gap (Kao and Bambara 2003). Interestingly, until the discovery of the flap endonucleases, it had been assumed that primer removal was mediated by ribonuclease-H, an RNAse specific for RNA that is H-bonded to DNA. Today, the consensus is that, if RNAse-H is involved at all, it only does so in collaboration with FEN1 (as discussed by Shultz et al. 2007).

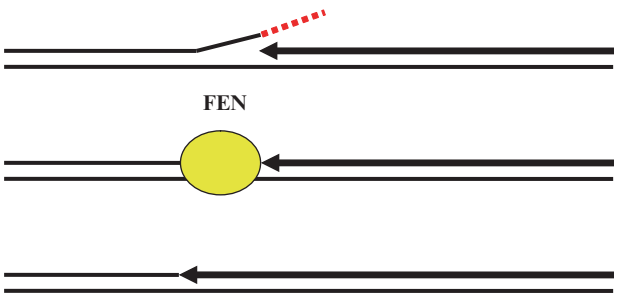

Fig. 2 Primer removal by Flap endonuclease: FEN1. The RNA part of the primer is indicated as a dotted line

6.6 Maturation: Joining the Pieces

The mechanisms of DNA synthesis just described leave the daughter strands as fragments. On the lagging side of the fork, the fragments are short – Okazaki fragments – but on the leading side, they are long, possibly as long as the complete distance from initiation site to terminus. Joining of the fragments is mediated by DNA ligase. In both budding yeast and fission yeast, there are two DNA ligases. The major ligase, DNA ligase 1, is encoded by *CDC9* in budding yeast (Barker et al. 1985) and *cdc17* in fission yeast (Johnston et al. 1986). In temperature-sensitive mutants of either of these genes, Okazaki fragments accumulate at the non-permissive temperature (reviewed by Bryant and Dunham 1988). In higher eukaryotes, which have several DNA ligases, it is also ligase 1 that joins the fragments (Bray et al. 2008).

Joining of Okazaki fragments occurs soon after synthesis but the joining of longer (replicon-sized) pieces is a slower process in plants and animals (reviewed by Van't Hof 1988). The process occurs in steps, going through phases in which replicons within a cluster are joined and then whole clusters are ligated to give the completed daughter strand. It is probable that this long-range joining process is dependent on the resolution of the daughter strands (see Sect. 6.6) and there is evidence from measurements of the size of nascent DNA strands that the completion of ligation may not occur until G2 (Schvartzman et al. 1981).

6.7 A Twist in the Tale

The progressive denaturation of DNA as the replication forks move outwards leads to DNA supercoiling in front of the forks. The current view is that the supercoiling is relieved by topoisomerase I, an enzyme that nicks one strand of DNA, allowing it to rotate around its partner strand and then reseals it. In topoisomerase I mutants, initiation of replication occurs normally but fork movement is strongly inhibited (see Bryant and Dunham 1988). A second type of topoisomerase, topoisomerase II is also involved. Unlike topoisomerase I, this enzyme requires ATP and mediates the nicking of both strands. It is required to resolve the daughter DNA strands at the points where replication forks of two adjacent replicons "back into" each other (Bryant and Dunham 1988).

7 Enzymes Involved in Plant DNA Replication

7.1 Replicative Polymerases: The DNA Polymerase-B Family

7.1.1 Introduction

In the current nomenclature, the replicative polymerases described above are classified (along with polymerase-ζ, involved in mutagenic DNA replication) as

B-family polymerases (Burgers et al. 2001). Biochemical, proteomic and genomic analyses indicate that plants possess all the B-family polymerases and that polymerases α and δ, (and probably polymerase-ε) are involved in DNA replication as described above.

7.1.2 DNA Polymerase-α

DNA polymerase-α has been purified from several species (reviewed by Bryant et al. 2001) and its close association with primase has been demonstrated in pea (Bryant et al. 1992), wheat (Benedetto et al. 1996) and tobacco (Garcia-Maya and Buck 1998). It is capable in vitro of initiation of synthesis on single-stranded templates and of extending primers on primed templates (Bryant et al. 1992, 2000). Indeed, in vitro, in the absence of primase and of the other replicative polymerases, it is often much more processive than when acting as the initiating polymerase in vivo. Its activity is highest in proliferating cells and lowest in cells in which DNA replication and cell division have ceased (e.g. Bryant et al. 1981). The cDNA encoding the catalytic subunit was first cloned from a rice cDNA library in 1997 by Yokoi et al., who showed that expression is regulated in a cell-cycle specific pattern. Genes encoding all four subunits have since been detected in the Arabidopsis and rice genomes (Shultz et al. 2007). More recently, the Arabidopsis *INCURVATA2* gene has been shown to encode the catalytic subunit of polymerase-α (Barrero et al. 2007). Strong mutant alleles are lethal; weak mutant alleles cause early flowering, leaf incurvature (hence the name of the gene) and homeotic transformations of floral organs. These authors ascribe these effects to an interaction between DNA replication and epigenetic patterning (see also Sanchez et al. 2008).

7.1.3 DNA Polymerase-δ

The characterisation of DNA polymerase-δ in plants was preceded by identification of its processivity factor, PCNA, first identified at gene level in 1989 (Suzuka et al. 1989). It was then shown that one of the DNA polymerase enzymes ("polymerase B") isolated from wheat was stimulated by PCNA (Richard et al. 1991); the stimulation was subsequently shown to be caused by PCNA acting as the polymerase's processivity factor. This was taken as evidence that polymerase B is in fact DNA polymerase-δ (Laquel et al. 1993). A cDNA encoding the catalytic subunit of polymerase-δ was isolated from a soybean cDNA library in 1997 (Collins et al. 1997); cDNAs encoding the catalytic subunit and one of the smaller subunits have subsequently been cloned from rice (Uchiyama et al. 2002). Genes encoding all four subunits are present in the rice and Arabidopsis genomes. The rice genome also has one PCNA gene, while Arabidopsis has two (Shultz et al. 2007). Both PCNA and polymerase-δ are expressed in dividing cells (Garcia et al. 2006; Kosugi and Ohashi 2002) and for PCNA, there is evidence that in synchronised

plant cell cultures expression is mainly confined to the S-phase (Kodama et al. 1991). In common with other proteins involved in DNA replication, the promoter of the PCNA gene has an E2F binding site (Kosugi and Ohashi 2002).

RFC (Replication Factor C), the PCNA loading factor, consists of five subunits. The first of these to be identified in a plant was the 36 kDa RFC3, cloned as a cDNA from rice (Furukawa et al. 2001). Its RNA was abundant in proliferating tissues and further, the level of transcription fell if cell division was inhibited. The same group then went on to clone the other four subunits. Expression of the whole RFC complex is related to cell proliferation, but there are interesting and subtle differences between the expression patterns of the five subunits (Furukawa et al. 2003). The rice genome contains one gene for each of the subunits as does that of Arabidopsis (Shultz et al. 2007).

7.1.4 DNA Polymerase-ε

At the time of writing this chapter, DNA polymerase-ε has not been unequivocally identified at the protein level as a specific, discrete enzyme activity in plants. However, genes encoding all four subunits have been identified in Arabidopsis and rice (Shultz et al. 2007). Indeed, Arabidopsis has two genes encoding the catalytic subunit, while rice has two genes encoding the largest of the regulatory subunits; both species have a family of genes encoding the smallest regulatory subunit. In Arabidopsis, disruption of one of the genes encoding the DNA polymerase-ε catalytic subunit (POL2a) or of the gene encoding the largest regulatory subunit leads to aberrant cell division patterns and to a sporophytic embryo-defective phenotype (Ronceret et al. 2005). Disruption of the second gene encoding the catalytic subunit (POL2b) has no effect, raising questions about the functionality of this second gene. The POL2a gene is also identified in Arabidopsis as the *TILTED* locus (Jenik et al. 2005). A viable mutation at this locus leads to a lengthening of the cell cycle by 35% throughout embryo development and to a change in cell type patterning, emphasising the interaction between regulation of the cell cycle and the determination of cell fate.

7.2 X-Family DNA Polymerases

The second mammalian DNA polymerase to be discovered was polymerase-β. It differs from polymerase-α in its response to inhibitors, its use of artificial template-primer systems and its co-factor requirements. It is a low molecular-weight enzyme, consisting of a single polypeptide chain and is much more tightly bound to chromatin than polymerase-α. Its distribution in relation to cell cycle activity suggested that it was not involved in DNA replication and it was widely regarded as a repair enzyme. This view has been confirmed by subsequent work

(Burgers et al. 2001), but there is more recent evidence that it may also have a role in DNA endoreduplication (Sect. 10).

For several years, there was discussion about whether plants possessed a polymerase-β-like enzyme. Some authors reported that they were unable to detect this type of polymerase, but others isolated and partially purified, from a range of dicot plants, an enzyme with very similar properties to the mammalian DNA polymerase-β (Dunham and Bryant 1986; Chivers and Bryant 1983; reviewed by Bryant and Dunham 1988). It was also suggested that a monocot, wheat, possessed a DNA polymerase-β-like enzyme (Laquel et al. 1993). After several years of debate between the protagonists of the two viewpoints, the topic faded from prominence as emphasis changed from traditional biochemistry to more molecular biological and genomic approaches.

Nevertheless, there has recently been renewed interest in this enzyme in plants. The identity of one of the wheat polymerases as a β-type enzyme has been confirmed (Luque et al. 1998). Tobacco cells contain an enzyme whose inhibitor responses resemble DNA polymerase-β (Quélo et al. 2002). A low molecular weight single polypeptide DNA polymerase has been purified from rice (Sanathkumar et al. 1996; Sarkar et al. 2004) and mungbean (Roy et al. 2007a, b, 2008). In the latter two species, its inhibitor responses are also characteristic of polymerase-β and furthermore, it is strongly and specifically recognised by antibodies raised against mammalian polymerase-β. The mungbean and rice enzymes are associated with DNA repair; indeed, the rice polymerase-β shows a direct functional equivalence to mammalian polymerase-β in its participation in base-excision repair. Further, although it was previously thought that Arabidopsis lacked a DNA polymerase-β gene (Burgers et al. 2001), a more recent annotation of the genome (MIPS 2008) has revealed the presence of this gene. The rice and *Medicago* genomes also contain genes that encode proteins with some sequence similarity to mammalian DNA polymerase-β (MIPS 2008).

Interestingly, there is some evidence that DNA polymerase-β is involved in DNA endoreduplication. If this suggestion is confirmed, it raises questions about the interaction between this polymerase and the other proteins involved in replication. This topic is discussed more fully in Sect. 10.

7.3 Other DNA Polymerases

Based on the most recent data, mammals are known to possess at least 15 DNA polymerases (Table 2). Discussion of the roles of these lies outside the scope of this chapter, except to note that mitochondrial DNA replication in mammals is mediated by polymerase- γ. Although orthologues of many of the mammalian polymerases are known from plants, the data do not provide evidence for the existence of polymerase-γ in *A. thaliana*. However, the *A. thaliana* genome does contain genes encoding two novel family-A enzymes similar to polymerase-θ, currently thought to be involved in replication of chloroplast and/or mitochondrial DNA

Table 2 DNA-dependent DNA polymerases in humans and in Arabidopsis[a]

Class/ Family	DNA Polymerase	Function	Human	Arabidopsis
B	α	DNA replication	√	√
B	δ	DNA replication	√	√
B	ε	DNA replication	√	√
B	ζ	Bypass synthesis	√	√
X	β	Base excision repair[b]	√	√[c]
X	λ	Base excision repair	√	√
X	μ	Non-homologous end joining	√	√
X	σ1	Sister chromatid cohesion	√	√
X	σ2	Sister chromatid cohesion	√	
X	TDT	Antigen receptor diversity	√	
Y	η[d]	Bypass synthesis	√	
Y	ι	Bypass synthesis	√	√
Y	κ	Bypass synthesis	√	√
Y	REV1	Bypass synthesis	√	√
A	γ	Mitochondrial DNA replication	√	
A	θ	DNA repair	√	√
A	θ-like1	Mitochondrial/Chloroplastic DNA replication?		√
A	θ-like2	Mitochondrial/Chloroplastic DNA replication?		√

Notes
[a]Based on the compilation by Burgers et al. (2001)
[b]May also have a role in DNA endoreduplication (see text: Sect. 10)
[c]Burgers et al. (2001) stated that Arabidopsis does not possess DNA polymerase-β. However, a gene encoding this enzyme has been located in a more recent annotation (see text: Sect. 7.2)
[d]Polymerases-η and -ι in humans are considered to be the result of a gene duplication

(Burgers et al. 2001; Mori et al. 2005a). It is probable the polymerases NtPolI-like1 and NtPolI-like2, recently discovered in tobacco BY2 cells, are the tobacco homologues of these enzymes (Ono et al. 2007).

8 Enzymes Involved in Maturation of the Replicated DNA

Maturation of the replicated DNA consists essentially of two phases, namely removing the primers and joining the tracts of nascent DNA. The first demonstration of flap endonuclease activity in plants was in cauliflower (Kimura et al. 1997). Although the mode of action was clearly described, the enzyme's role was not understood at that time. It has since been characterised at protein, cDNA and gene levels in rice (Kimura et al. 2000, 2001, 2003). Expression, assayed at both transcript and protein levels is mainly confined to meristematic regions (Kimura et al. 2000, 2001, 2003), but also occurs in *Lilium* micro-sporocytes during meiosis

(Kimura et al. 2001) The rice genome contains two copies of the FEN1 gene (Kimura et al. 2003), whereas the Arabidopsis genome contains one (Shultz et al. 2007).

Ribonuclease-H was first isolated from plant cells in 1979 (Sawai et al. 1979) with evidence that there was more than one isoform. An enzyme exhibiting RNAse-H activity was detected in a protein complex associated with DNA-polymerase-α-primase in pea (Bryant et al. 1992). Genomic analysis shows that neither the Arabidopsis genome nor the rice genome contains RNAse-H1 coding sequences but that they both possess an RNAse-H2 gene (Shultz et al. 2007). It is presumed that the enzyme activities previously detected represented RNAse-H2.

In contrast to the relative paucity of data on the enzymes of primer removal, much more is known about the enzymes that join pieces of DNA, the DNA ligases. As reviewed recently by Bray et al. (2008), plants contain at least three (and in Arabidopsis possibly four) genes encoding DNA ligase. As in other eukaryotes, DNA ligase1 is the enzyme responsible for joining the fragments generated during DNA synthesis (Taylor et al. 1998; Wu et al. 2001) and for the ligation of *Agrobacterium* T-DNA into plant genomic DNA (Ziemienowicz et al. 2000). In most eukaryotes, the DNA ligase1 transcript contains two open reading frames. Thus, one mRNA encodes two enzymes, one targeted to the nucleus and one to the mitochondria (Bray et al. 2008). The plant DNA ligase1 transcript contains three possible reading frames and it is tempting to suggest that the extra reading frame (in comparison with the yeast or the animal ligase 1) encodes a chloroplast-directed enzyme; but at present, the experimental evidence does not support this (Bray et al. 2008; Sunderland et al. 2006).

In mammals, the localisation of DNA ligase 1 to the nucleus during S-phase and its interaction with PCNA (which aids its binding to the nick sites) is regulated by phosphorylation: The un-phosphorylated form is the form active in S-phase. At present, there is no evidence that this is true of plant DNA ligase 1 (Bray et al. 2008).

9 Untwisting Plant DNA

Topoisomerases I and II have both been purified from a range of plant species (reviewed by Singh et al. 2004), including pea (Chiatante et al. 1993), tobacco (Heath-Pagliuso et al. 1990), carrot (Carbonera et al. 1988), cauliflower (Fukuta et al. 1986) and Arabidopsis (e.g. Makarevitch and Somers 2005). In pea, a sub-population of topoisomerase I is associated with DNA polymerase-α-primase (Bryant et al. 1992). In general, activities of both are higher in proliferating cells than in non-proliferating cells (Balestrazzi et al. 1996; Singh et al. 2004; Xie and Lam 1994). A cDNA encoding Arabidopsis topoisomerase I is able to rescue topoisomerase I mutants of budding yeast (Kieber et al. 1992). The latter authors also obtained evidence suggesting that the Arabidopsis genome contained just one topoisomerase I gene. However, there are actually two genes (topoI-alpha and topoI-beta), which are adjacent to each other (Takahashi et al. 2002). Intriguingly,

mutations in the alpha gene cause aberrant phyllotaxis (Takahashi et al. 2002). Inhibition by RNAi of the beta gene in plants already carrying the mutant alpha gene is lethal, demonstrating that topoisomerase 1 is essential. As with topoisomerase I, cDNAs encoding topoisomerase II have been cloned from several species (Singh et al. 2004); the tobacco cDNA rescues successfully a temperature-sensitive topoisomerase II mutant of budding yeast (Singh et al. 2003).

10 DNA Endoreduplication

In the discussion of activation or licensing of origins (Sect. 5.3), it was emphasised that there are multiple mechanisms to ensure that this happens only once per cell cycle (see Arias and Walker 2007 and Tanny et al. 2006 for a fuller discussion). However, DNA endoreduplication, in which DNA replication is repeated, often several times, without an intervening mitosis, is very common in plants. The plant cell is thus able to bypass the signals and checkpoints that normally prevent entry into S-phase unless mitosis has been completed. The mechanisms that operate at organ, tissue and cell level to allow this breakage of the normal rules have been recently reviewed (Larkins et al. 2001; Francis 2007; Caro et al. 2008) and are not discussed further here. Instead, attention is focussed on two of the molecular events.

Firstly, in order to initiate any form of DNA replication, a pre-replicative complex must be formed. The key role of CDC6 in this, and its regulation both at the transcriptional level and by protein degradation, has been noted (Sect. 5.3). It is now clear that CDC6, and to a lesser extent CDT1, are important factors in endoreduplication (Castellano et al. 2001, 2004) In Arabidopsis, the CDC6 protein is present both in cells undergoing DNA replication in a normal cell cycle and in cells undergoing DNA endoreduplication. In the latter, however, the rapid destruction of the protein after S-phase does not occur, so that CDC6 has a much longer half-life. Further, ectopic expression of CDC6 induces DNA endoreduplication in plants, while recruitment of CDC6 to DNA is sufficient to set up an artificial origin of replication in mammalian cells (Takeda et al. 2005). Over-expression of CDC or CDT1 leads to DNA "over-replication" in mammals, as does depletion of the mammalian CDT1 inhibitor, geminin (Dorn et al. 2009). In cells in which endoreduplication occurs some time after normal cell cycle activity has ceased, CDC6 expression is renewed. This is seen in tobacco BY2 cells (see Fig. 3) and also in mammalian cells (Bermejo et al. 2002).

The second point concerns the role of DNA polymerases. It is expected that the biochemistry of DNA synthesis will be the same in DNA endoreduplication as in normal DNA replication. Indeed, the participation of the pre-replication complex in endoreduplication reinforces this expectation. However, as long ago as 1982, it was shown that in rat trophoblasts, DNA endoreduplication can be brought about by DNA polymerase-β (Siegel and Kalf 1982). A similar conclusion was reached by Quélo et al. (2002). The latter group showed, very unexpectedly, that in cultured

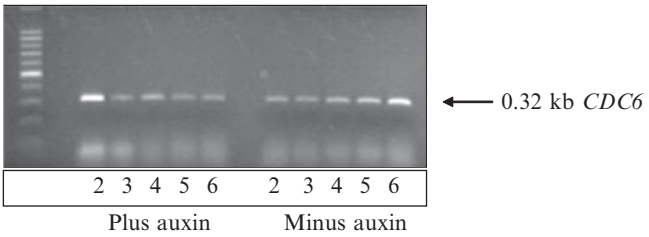

Fig. 3 Expression of *CDC6* in tobacco BY2 cells sub-cultured in the presence or absence of auxin. In the presence of auxin, the cells re-initiate cell division and reach log phase at 2 days; cell division rate then declines and the cells enter stationary phase. In the absence of auxin, cell division is not reinitiated but the cells start to endoreduplicate their DNA at day 4 to 5 and remain active through day 6 and beyond. *CDC6* expression was monitored by RT-PCR amplification of a specific fragment of *CDC6* mRNA. Details of primers and PCR conditions are given in Dambrauskas et al. (2003).Unpublished data of A-H Quélo, J-P Verbelen, G Dambrauskas, SJ Aves, JA Bryant, HJ Rogers and D Francis

tobacco cells, DNA endoreduplication still occurred when the replicative DNA polymerases were inhibited and only ceased if an inhibitor of DNA polymerase-β was also applied. However, their data still leave room for the participation of the replicative polymerases: Cultures in which only polymerase-β was inhibited were able to undergo DNA endoreduplication. More recently, Roy et al. (2007a, 2007b, 2008) have extracted, purified and characterised a DNA polymerase-β from mungbean (as discussed in Sect. 7.2). In developing mungbean seeds, it appears to be specifically associated with cells undergoing endoreduplication (Roy et al. 2007a, b). Indeed, these were a good source for enzyme extraction and purification. Further, this polymerase-β-like enzyme is capable of interacting with PCNA, thereby increasing significantly its processivity (Roy et al. 2007a). This makes a role in DNA endoreduplication more feasible.

11 Concluding Remarks: Looking Backwards and Forwards

Knowledge of plant DNA replication has come a long way since I started my research on the topic. At that time, the field was in its infancy. A tiny handful of laboratories, including my own, were investigating the enzymology of DNA replication, especially the DNA polymerases (e.g. Dunham and Cherry 1973; Robinson and Bryant 1975; Stevens and Bryant 1978). Amongst eukaryotes, most progress on the enzymes of DNA metabolism was being made in research on vertebrates, leading to the compilation of a list of different enzyme activities needed to replicate a DNA molecule. Work on plants lagged behind somewhat, but by the early 1980s, it was possible to state that all the enzymes in the list had been detected in plants; however, most had not been well characterised (see, e.g. Bryant 1986). Further, even for vertebrates, there was no real clue as to how many DNA polymerases they actually possessed.

One of the most pressing questions, both for animals and plants, was the recognition by the DNA replication enzymes of the origins of replication that were clearly visualised (although not characterised) by fibre autoradiography. To some extent, many of us working on eukaryotic DNA replication were "distracted" in this respect by a model system, the replication of simian virus 40 (SV40). When SV40 replicates in the cells of its mammalian hosts, strand separation is initiated by the binding of one protein, T-antigen (albeit acting as an oligomer) to the replication origin. It is the only virus-encoded protein involved in the replication of the viral DNA; host proteins do the rest. The search was thus "on" for a host cell equivalent of T-antigen, i.e. a protein that would recognise and bind to replication origins and then initiate strand separation. As already indicated, this search was at that time unfruitful. It was not the early 1990s that any real progress was made in respect of origin recognition and I have already noted that the work of Bell and Stillman (1992) was a real turning point for this research.

In the meantime, there had been increasing focus since the early 1980s on other eukaryotic model organisms, namely budding yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). In both these organisms, there is a large range of temperature-sensitive mutants. Many of the mutations directly affect progress of the cells through the cell division cycle, thus leading to the identification of a range of cell-division cycle (*CDC* or *cdc*) genes and of genes that interact with the *CDC*/*cdc* genes. Our knowledge of the proteins involved in DNA replication has thus expanded rapidly, revealing, as indicated in the text of this chapter, a level of complexity undreamed of 20 years ago. Further, because of advances in immuno-detection, gene sequencing and bio-informatics, it has proved possible to quickly compare other organisms with the yeasts, thus circumventing the relative scarcity of suitable mutants in diploid organisms. This in turn has accelerated the acquisition of knowledge about plant DNA replication so that we can, as demonstrated by Shultz et al. (2007), be confident that the general picture built up for other eukaryotes also applies to plants. There are still some unanswered questions. How many more proteins will be discovered that act at the replication origin or at the replication fork? How exactly does the origin-recognition complex recognise the origins? Work on these and other facets of DNA replication will certainly be helped by the establishment of two databases, one devoted to replication origins (Nieduszynski et al. 2007) and the other to more general molecular aspects of replication (Cotterill and Kearsey 2009).

So, what are the future directions for research on plant DNA replication? I will focus on some of the topics that I consider important. They are all related to the understanding of the plant-specific mechanisms that regulate the universal molecular events. Firstly, there are several developmental situations in which the rate of DNA replication and hence, the length of S-phase changes. The data suggest that fork rate can change (e.g. Jacqmard and Houssa 1988).This in itself is intriguing, raising the possibility that enzyme activities themselves can be regulated in relation to development. The data also show conclusively that the number of origins that are used can be altered, sometimes quite dramatically (as discussed in Sect. 4). Developmental cues thus alter the activation of origins, but at exactly which point in the process is unknown.

Secondly, DNA replication is normally embedded with the cell division cycle, which in turn is regulated by cyclins, cyclin-dependent kinases, phosphatases and checkpoint mechanisms. In relation to the events discussed in this chapter, there must be a range of interactions that regulate S-phase proteins at both transcriptional and post-transcriptional levels. Some of these we know about, such as the RB-E2F-DP system for transcriptional regulation (see e.g. Menges and Murray 2008), but there is much more to discover here.

Thirdly, there is the widespread occurrence in plant development of over-riding the "once-per-cycle" licensing system for DNA replication. DNA endoreduplication (Sect. 10) is a common phenomenon in plants and is a feature of development in many different cells and tissues. It is my contention that there is still much to discover about this. Certainly it is true that cells can over-ride the controls that normally regulate passage through M-phase (Francis 2008), but there must still be a mechanism to ensure that DNA replication is initiated. The role of CDC6 is important here and it is fascinating that whether its expression promotes an S-phase within a normal cell cycle or within an endocycle depends on cell type (Caro et al. 2008). Thus, factors specifying cell identity can interact with the molecular machinery of DNA replication in a cell-type-specific manner, but the specific mechanism by which this is achieved is not known. Further, there are the unexpected findings that DNA polymerase-β may participate in DNA endoreduplication (Sects. 7.2, 10), which raises questions about how it interacts with the origin-activation mechanisms described in this chapter.

Finally, there is the very important topic of the regulation of DNA replication, whether in a "normal" cell division cycle or in endoreduplication, in relation to development. The importance of cell type was mentioned immediately above. The specification of cell, tissue and organ identity is central to the development of multi-cellular organisms. In relation to cell division, the focus is on the establishment and maintenance of meristems via the expression of meristem identity genes. One of their downstream effects is on the activity of genes encoding the enzymes and other proteins involved in DNA replication and we are beginning to understand how this regulation is achieved. However, it is becoming apparent that there are more subtle interactions between tissue patterning and DNA replication proteins. Thus, disruption or inhibition of certain DNA replication proteins may have developmental effects that go beyond a straightforward inhibition of the cell cycle, as exemplified by MCM7 (Springer et al. 1995, 2000), DNA polymerase-α (Barrero et al. 2007) and topoisomerase I (Takahashi et al. 2002). We may conclude that plant DNA replication and its control remain very fruitful topics for research.

Acknowledgements I must express my gratitude to the DNA replication group at North Carolina State University, Raleigh. Their genome-wide analysis of the genes and proteins involved in plant DNA replication (see Shultz et al. 2007) is of great value to the plant cell cycle research community and saved me much time in writing parts of this chapter.

I am grateful to all the postgraduate students, post-doctoral researchers and visiting scientists who have worked in my lab on aspects of DNA replication and to those colleagues at Exeter and elsewhere with whom I have been able to collaborate. I especially thank Steve Aves and Dennis Francis for long-term working relationships and their ready willingness to discuss DNA replication

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Transformations of Cellular Pattern: Progress in the Analysis of Stomatal Cellular Complexes Using L-Systems

Peter Barlow and Jacqueline Lück

Contents

Abstract Stomatogenesis involves a progressive transformation of selected protodermal cells on primary plant tissue surfaces, especially those of leaves, and culminates in the formation of a functional stomatal apparatus. The transformations are accompanied by species-specific stereotypical productions of cells, commencing with an asymmetric cell division which produces a meristemoid having a prescribed number of future divisions and concludes with a symmetric division that produces a pair of guard-cells. Where all cells of the stomatal cellular complex descend from a meristemoid, and where subsidiary epidermal cells are produced from intervening steps, the pathway is said to be mesogenous. It is often found in dicot plants. In contrast, the perigenous pathway often consists of fewer divisions of the meristemoid

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and incorporates into the ontogeny of the stomatal complex one or more of the protodermal cells which neighbour the meristemoid; it is their asymmetric divisions which produce subsidiary cells. This pattern is often found in monocots. The auto-reproductive meristemoidal state may, in certain cases of the mesogenous pathway, be perpetuated by means of a stomatogenic branching process in one of the subsidiary cells, as shown by the formation of "satellite" stomatal cellular complexes on leaves of *Arabidopsis thaliana*. L-system algorithms are developed that prescribe not only the cell divisions and transformations but also the stomatogenic cellular patterning that occurs throughout the angiosperms. Mutations of the patterning process, and natural variations, such as stomatal clustering, are also discussed. All the division patterns (normal, mutant, and unusual) necessary for the structuring of leaf stomatal complexes, including examples of the so-called "one-cell rule" of stomatal spacing, can be modelled by an appropriate deterministic L-division system. Because the division systems can be analogised to states of the wall and peripheral cytoplasm that attract the attachment of new division walls, this cytological aspect of meristemoids would appear to deserve more attention. Gene regulation is an additional component of stomatal construction. Such processes help to initiate the cytological analogues that are reproduced by L-systems within a framework composed of hitherto uncommitted protodermal cells. Gene regulation also terminates the auto-reproductive property of the division system and leads to the differentiation of the cells which construct the stomatal complexes.

1 Introduction

An interesting question is whether present-day biology needs a theoretical "substratum" from which modelling emerges as an indispensible component of research strategies. Although today many would think it rash to embark on writing a purely theoretical biology text, 80 or so years ago, Jakob Von Uexküll, Joseph H Woodger and Ludwig Von Bertalanffy were doing just that, as well as making other important practical contributions in their respective fields. Working from different biological perspectives, their publications (Von Uexküll 1928; Woodger 1929; Von Bertalanffy 1932) sought for a communicational method whereby biological systems could have an objective representation. Deploring the limitation of language and the concepts that were necessarily borne by it, Woodger (1945) remarked that: "We still struggle on with the same linguistic equipment long after the accumulation of facts has ceased to be accommodated by it. Who... has not felt the difficulty of finding adequate verbal expression for something which has seemed tolerably clear in thought?"

In recent years, the ideas of all the three aforementioned authors have been revivified: Von Uexküll's work has become central to the emerging field of biosemiotics (Barbieri 2008), Von Bertalanffy's "systems biology" has seen a recrudescence (Kell and Oliver 2004; Aderem 2005), while Woodger's ideas were taken up in the 1960s by Aristid Lindenmayer, his colleague at University College, London, and

developed into that author's L-systems,[1] which have since found applications not only in biology and computing science (Lindenmayer and Rozenberg 1976; Rozenberg and Salomaa 1980) but have also been adopted in other unexpected areas (McCormack 2003, Worth and Stepney 2005).

The inception of L-systems was influenced by Woodger's idea of "taxonomic transformation" (Woodger 1945). Woodger believed that, "given a certain sort of zygote and a certain sort of environment", it might be possible to devise a theory by means of which "we can predict the sort of time-slice that will be realised at a given time" – in other words, a suitable theory of development would predict the form of an organism at any time in the future. Woodger suggested that the morphological transformations within an evolutionary timescale (which in turn would lead to the construction of a taxonomy) would necessarily be accompanied by corresponding genetic transformations, and that both types of transformation would be governed by rules inherent to the corresponding biological system, so that "all taxonomic transformations will be immediately deducible from the embryological and genetic ones" (Woodger 1945). The transformational systems and the rules that specify them would thereby constitute the theoretical strand which Woodger et al., had sought, and which would rationalise both data-collecting projects and hypothesis-driven experimental lines of research. Biology might then be seen as a tripartite system comprised of: (1) genetic networks and their transformations, (2) metabolic and functional state networks and transformations, and (3) cellular and morphological transformations. Needless to say, there would also have to be a means of linking these three systems together.

The present chapter surveys, primarily, the types of transformations and rules, which apply to the cellular development of stomatal complexes. Genetic networks (systems of genetic transformations) run in parallel with these ontogenetic, or transformational, pathways and some attention is paid to them, where they impinge on our primary subject matter of cellular constructions. These, in turn, would lead to a taxonomy of stomatal complexes. The processes needed for "spacing out" stomata on a leaf surface are also important, but we have not delved too deeply here. However, we have been keen to investigate the extent to which cellular interactions seem to be necessary in the spacing of stomatal complexes. Our preoccupation has been with Lindenmayer-type division systems, and so we have tended to examine whether these systems, here based on states of the physico-chemical boundary of the cells, are alone sufficient to account for the cellular patterns and stomatal complex distributions on a leaf surface.

The cellular transformations proposed for the genesis of stomatal complexes constitute the third of the three research strands mentioned above. Regarding the other two strands, stomatal genetics and networks have been reviewed by Benítez

[1] It has been said that Aristid Lindenmayer was one day passing a lecture room in which a lecture about formal languages was taking place. The speaker mentioned L(G): "Algae!" was Lindenmayer's immediate reaction. After this "eureka moment", he started to investigate applications of language theory to developmental biology. Algae constituted the first example (from Rozenberg and Salomaa 1986, p. 383–384).

et al. (2007), and stomatal metabolism and functional regulatory networks, especially in relation to the guard-cells, have been reviewed by Fan et al. (2004), Li et al. (2006), and Soni et al. (2008). Thus, the foundations of a tripartite structure for stomatal biology are already in place. This indicates that the stomatal complex can be held up as an example of a truly model functional plant organellar (in the sense of a small organ) system.

2 Transformations of Cellular Pattern

In this and the next section, L-systems are introduced. They have been employed here to simulate the pathways of stomatal complex ontogeny. Then, in Sects. 4 and 6, correspondences are made between L-systems and some of the known types of stomatal complexes. The ramifications of these applications of L-systems to stomatal complex formation are explored in the remaining sections.

In the initial stages of their development (Lindenmayer 1971, 1975; Lindenmayer and Rozenberg 1976), L-systems were employed to simulate cellular and morphological transformations. Examples studied in the plant kingdom ranged from one-dimensional algal filaments (Lück and Lück 1976; Barlow and Lück 2008) to the three-dimensional patterns of inflorescence development and sequences of flower opening and fading (Prusinkiewicz and Lindenmayer 1990). Many of these examples were analysed using the class of systems referred to as D0L-systems: that is, the L-system in question is determinate (D) and does not depend on any external input (0). D0L-systems require the assignment of labels to structures (say, walls and edges of a cell or an organ). Then, over a series of timesteps, the labels are transformed according to a set of rules. The states (or structures) which the labels represent are also transformed. Not only are the D0L-systems powerful in analysing morphogenesis, but they can also be applied in other ways, such as unravelling the cell production events that determine the characteristic distributions of cell lengths along the cell files of root meristems (Lück et al. 1994). Extensions of D0L-systems are map systems (M0L-systems). These can simulate the growth of cellular arrays in two-dimensions, where regular hexagonal "cells" are employed as a starting point (Lindenmayer and Rozenberg 1979; Abbott and Lindenmayer 1981). The simulated isotropic growth of these hexagonal arrays conforms to the growth observed in cells on planar surfaces, such as in certain areas of leaf epidermis (Lück et al. 1988).

The attachment of labels to both sides of a wall shared by two cells provides a labelled double wall (dw). The corresponding dwM0L-systems (Lück and Lück 1981, 1985, 1987) are then able to achieve a great range of morphological outputs, in the form of a double wall map, including the simulation of the three-dimensional "cell-works" of meristematic apices (Lück and Lück 1981, 1991). Conformity of labels of each double wall enables the assemblage of cells to behave as one continuous developmental unit. Moreover, the cellular assemblage may naturally give rise to cellular units differentiated by virtue of their growth properties (Lück and Lück 1987). In one particular case, from the

starting point of a three-sided cell[2] and with the application of an appropriate dwM0L-system, ten different cellular states emerged, via an L-system algorithm, over five timesteps (Barlow and Lück 2004a). The edges of the various cells were organised so that differential cell growth arose, as well as reversals of handedness of the cell productions. In the process of map development, inert growth zones developed: some of these had a cell pattern resembling that of a "quiescent centre", as is found in roots, for example (Barlow and Lück 2004a; Barlow et al. 2004a), or in the notches of shoot buds (Lück and Lück 1993).

3 L-Systems Applied to Stomatal Cellular Complexes

We shall now explore the application of L-system-based transformations to the ontogeny of the stomatal complexes of angiosperms. A first attempt at this has already been made by Barlow and Lück (2007). The analysis of the stomatal cellular complexes is confined to two organisational levels and uses the appropriate L-system: (1) a D0L-system is applied at the level of cells, which construct the complex; (2) at the level of the elements (edges or wall segments), which construct the cells, dwM0L-systems apply. Together, these systems indicate the exact spatial relationships of the cells.

Both types of system (D0L or dwM0L) comprise: (a) an alphabet of state labels, which apply to the elements from which each type of construction is derived, (b) a set of production rules, which governs the transitions of state labels, and (c) an initial word, ω_0. Transition events occur synchronously at discrete timesteps. In the case of D0L-systems, where cell state labels are used, the transitions are of the form $A \rightarrow B$ or $A' \rightarrow B'\ C'$, the arrow ($\rightarrow$) indicating a transformation event. In the first case ($A \rightarrow B$), the product consists of just one label (B) due to a state change; in the second case ($A' \rightarrow B'\ C'$), the product has two labels because of the binary division of the cell A'. In the present context, the initial word ω_0 refers to a meristemoid,[3] a cell derived from a leaf protodermal cell and from which cells of the stomatal complex are descended. In the case of dwM0L systems, the wall alphabet corresponds to half-wall segment labels. The product, B, of their transitions can be

[2]The "sides", or "edges", of a cell are those seen in two-dimensional plan view, as might be observed in a microscope. In their three-dimensional context, the "sides" are actually "side walls" or "wall facets".

[3]Meristemoids have been variously defined, but generally they are cells capable of renewed meristematic activity (of "reactivated embryonality", as Bünning (1956, 1965) put it when he introduced the term "meristemoid"), which leads their descendents to a fate different from that of neighbouring cells. In Bünning's writings (Bünning 1956, 1965), emphasis is placed on a hypothetical inhibitory field emanating from each meristemoid and which is believed to regulate the positioning of new meristemoids (see Sachs 1991); but this physiological aspect is supplementary to the more anatomically based definition provided here. Before a meristemoid is produced, there is a polarisation of cytoplasm in the mother cell; this cell subsequently divides asymmetrically to produce one meristemoid containing the dense polarised cytoplasm and another type of daughter cell (See also footnote 11).

composed of several elements which are separated either by division wall insertion nodes (/) or by virtual nodes. Slashes specify attachment sites for a new wall when a mother cell divides. Virtual nodes are not explicitly indicated but they have a role in the regulation of wall growth. A complete sequence of cell segments surrounding a cell can be concisely represented by a cycle of segments, for which corresponding transformations occur during a timestep. This cycle of segments is equivalent to the cell alphabet, although with the addition of cellular spatial relationships. The transformations lead to the various types of stomatal complexes listed in Table 1 (see Sect. 4.4).

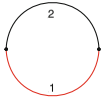
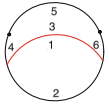
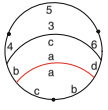
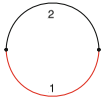
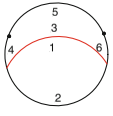
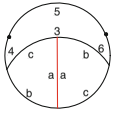
4 Cells of the Stomatal Complex and Their Transformations

Stomata are formed within the protodermal cell layer of young leaves, usually on the abaxial and adaxial surfaces, but sometimes on only one of them, frequently on the abaxial surface (Cutter 1971; Evert 2006). The sequence in which the stomatal complexes are formed is either basipetal, acropetal or (occasionally) simultaneously over the whole leaf surface to give a patchwork of stomata (Pant 1965). On account of their cellular patterns and ontogenies, the complexes were classified into 27 types by Fryns-Claessens and Van Cotthem (1973). These types are used as points of reference in our examination of stomatogenesis.

4.1 Types of Cells

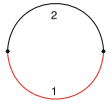
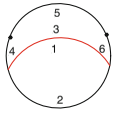
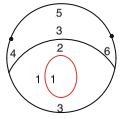
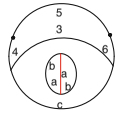
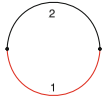
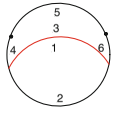
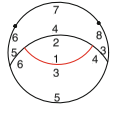
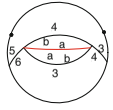
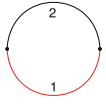
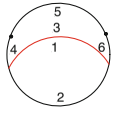
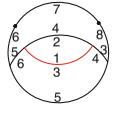
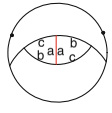
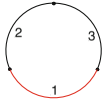
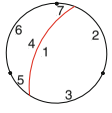
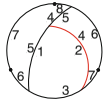
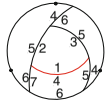
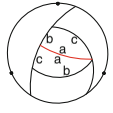
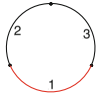
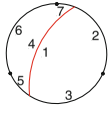
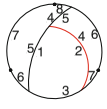
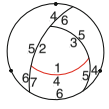
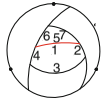
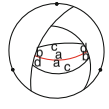
Stomatal complexes of dicots commence their development during a specific phase of leaf growth. Each complex is initiated by an unequal division of a protodermal cell. The smaller daughter cell is an auto-reproductive "meristemoid" (stomatal complex mother cell), designated here as m_0. The larger daughter cell continues as a protodermal cell (P), which later differentiates as an epidermal cell (E). Whereas in many dicots the subsidiary cells (S) which neighbour the guard-cells (g) are descendents of m_0, in the case of monocots S cells are often induced to form, following a cell division, from P cells which are neighbours of the meristemoid. S cells are generally believed to be distinct and differentiated from P, E and g cells. Morphological and histological criteria have helped to define the S cells of dicot and fern stomatal complexes (Patel et al. 1975; Galatis 1977; Patel 1978), while in monocots, the metachromatic stain toluidine blue has proved useful in distinguishing S cells from other types of neighbouring cells (Gallagher and Smith 2000). Galatis (1977), however, has suggested that, with respect to their internal structure, the S and g cells (of *Vigna sinensis*) are by and large the same, except (of course) for their different shapes. S cells, in their turn, may make contact with distinctive "encircling" cells (N) (Pant 1965).

Table 1 Summary of stomatal cellular complexes and their dwM0L-division systems. The nomenclature of the stomatal-complex types is from Fryns-Claessens and van Cotthem (1973)

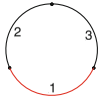
Type number and Stomatal type	Meristemoid m_0 and its wall labels (initial word ω_0)	Division system	Number of formative divisions (λ) producing S cells, which surround a pair of guard-cells				Guard mother cell (m_λ) division	Comments (λ values, number of S cells, genus and species in which example is found, etc.)
			1	2	3	4		
1 Agenous Perigenous		(1 2) → (c/$_a$b c/$_a$b)						$\lambda = 0$ 0 S
17 Euperi-Mesogenous		(1 2)→ (2 3) + (1/$_1$) (1) → (b/$_a$b/$_a$) (2 3) → (2 3)						$\lambda = 1$ 1 S Insular *Anemia*
9a Meso-Perigenous		(1 2) → (6/$_1$2/$_3$4 5) (1 2) → (b c/$_a$b/$_a$) (3 4 5 6) → (3 4 5 6)						$\lambda = 1$ 1 S *Ranunculus*
9b Hemipara- Meso-Perigenous		(1 2) → (6/$_1$2/$_3$4 5) (1 2) → (c d/$_a$b/$_a$b) (3 4 5 6) → (3 4 5 6)						$\lambda = 1$ 1 S Paracytic *Tetracentron*
10 Eupolo- Meso-Perigenous		(1 2) → (6/$_1$2/$_3$4 5) (1 2) → (c/$_a$b c/$_a$b) (3 4 5 6) → (3 4 5 6)						$\lambda = 1$ 1 S Diacytic *Plagiogyria*
18 Coperi-Mesogenous		(1 2) → (6/$_1$2/$_3$4 5) (1 2) → (2 3) + (1/$_1$)						$\lambda = 2$ 2 S

(*continued*)

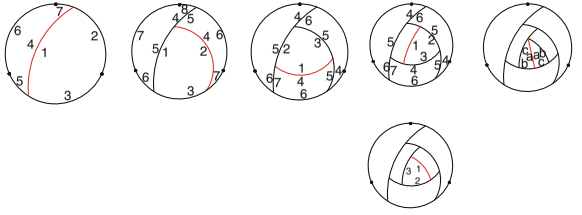
Table 1 (continued)

Type number and Stomatal type	Meristemoid m_0 and its wall labels (initial word ω_0)	Division system	Number of formative divisions (λ) producing S cells, which surround a pair of guard-cells				Guard mother cell (m_λ) division	Comments (λ values, number of S cells, genus and species in which example is found, etc.)
			1	2	3	4		
		(3 4 5 6) → (3 4 5 6) (1) → (b/$_a$b/$_a$) (2 3) → (2 3)						1 Helico-1 Insular-development goes from Type 9a to Type 17. *Pyrrosia*
21 Para-Mesogenous		(1 2) → (6/$_1$2/$_3$4 5) (3 4 5 6) → (345 6 7 8) (1 2) → (b/$_a$ b/$_a$) (3 4 5 6) → (3 4 5 6) (3 4 5 6 7 8) → (3 4 5 6 7 8)						λ = 2 Non-deterministic! Paracytic Rubiaceous
8 Dia-Mesogenous		(1 2) → (6/$_1$2/$_3$4 5) (3 4 5 6) → (345 6 7 8) (1 2) → (3/$_1$2 3/$_1$2) (3 4 5 6 7 8) → (3 4 5 6 7 8)						λ = 2 2 S 2 Helico-Mesogenous Mesodiacytic
24a Aniso-Mesogenous		(1 2 3) → (3/$_4$5 6 7/$_1$2) (4 5 6 7) → (45 6 7 8) (4 5 6 7 8) → (4 56 7 8 9) (1 2 3) → (b c/$_a$b c/$_a$) (4 5 6 7 8 9) → (4 5 6 7 8 9)						λ = 3 3 S Helico-Mesogenous Anisocytic Cruciferous *Arabidopsis thaliana*
24b Aniso-Mesogenous		(1 2 3) → (3/$_4$5 6 7/$_1$2) (4 5 6 7) → (45 6 7 8) (4 5 6 7 8) → (4 56 7 8 9) (1 2 3) → (3 4/$_5$6 7/$_1$2) (1 2 3 4) → (c d/$_a$b c d/$_a$2) λ = 4: new wall out of sequence (cf. Type No. 25)						λ = 4 4 S 4 Helico-Mesogenous *Vinca*

25
Helico-Mesogenous



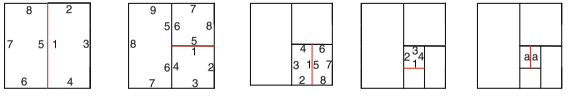
(1 2 3) → (3/$_{4}$5 6 7/$_{1}$2)
(4 5 6 7) → (45 6 7 8)
(4 5 6 7 8) → (4 56 7 8 9)
(4 5 6 7 8 9) → (4 5 6 7 8 9)
(1 2 3) → (b c/$_{a}$b c/$_{a}$b)



λ = 4 or 5
4 or 5 S
Kalanchoë

26a
Tetra-Mesogenous

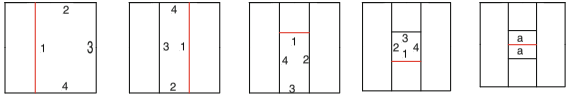
(1 2 3 4) → (4/$_{5}$6 7 8/$_{1}$2 3)
(5 6 7 8) → (45 6 7 8)
(5 6 7 8 9) → (6 7 8 9 0)
(6 7 8 9 0) → (6 78 9 0 A)
(1 2 3 4) → (d/$_{a}$b c d/$_{a}$b c)
(6 7 8 9 0 A) → (6 7 8 9 0 A)



λ = 4
4 S
4 Labrate-Mesogenous
or 4 Helico-
Mesogenous

26b
Tetra-Mesogenous

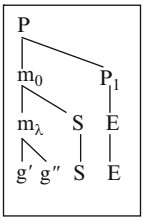
(1 2 3 4)^{1} → (4/$_{5}$6 7 8/$_{1}$2 3)
(1 2 3 4)^{2} → (3 4/$_{5}$6 7 8/$_{1}$2)
(1 2 3 4)^{3} → (8/$_{1}$2 3 4/$_{5}$6 7)
(1 2 3 4)^{4} → (3 4/$_{5}$6 7 8/$_{1}$2)
(1 2 3 4) → (c d/$_{a}$b c d/$_{a}$b)



λ =4
4 S
? Division sequence
North South
East West
Tradescantia, Allium

The initial word, ω_0, indicates the cycle of wall states of the meristemoid, m_0. All walls are numbered with their state labels, in clockwise direction, before and after their step-wise transformation (in this case, formative divisions 1, 2, 3 or 4). The division system by which each stomatal type is produced commences after the establishment of m_0 and its initial word ω_0. The state transformations are specified by the arrow (→). The most recent division walls are denoted by red lines. Notice that the state of the wall of each meristemoid is conserved in the m_i descendents through λ successive divisions, as befits cells with autoreproductive stem-cell status. After these divisions, the guard mother cell, m_λ, divides. The orientation of this final division with respect to the preceding division corresponds to θ_g, thereby producing either paracytic, diacytic or anomomeristic types of stomata. All S cells are mesogenous, but not all of them retain contact with the guard cells. The ordering of the stomatal types in the left-hand column is not precisely in the numerical sequence offered by Fryns-Claessens and Van Cotthem (1973). Rather, the sequence accords with the logic of the division system presented in the next column. Further details of the division-system conventions are in Lück and Lück (1985).)

Not all the cells making contact with a pair of g cells can be distinguished specifically as S cells; many may be regular epidermal E cells. In the case of *Arabidopsis thaliana*, Yang and Sack (1995), for example, prefer not to call the larger cells, which descend from a meristemoid m_0, S cells, believing them not to meet the criteria that would set them apart from E cells. One possible general descendence for stomatal complex cells of dicots (in which S cells are recognisable) is shown as:



Or, at right, alternatively notated as a set of cell productions:

$$P \rightarrow m_0\ P_1$$

$$m_0 \rightarrow m_\lambda\ S$$

$$m_\lambda \rightarrow g'\ \ g''$$

$$P_1 \rightarrow E \rightarrow E$$

$$S \rightarrow S$$

$$g'\ \ g'' \rightarrow g'\ \ g''$$

Visual identification of the type(s) of cells which surround the g cells, having been produced by the mesogenous pathway, is evidently a problem. One function of the S cells (as seen in monocots) is to exchange water and K^+ ions with the g cells and thereby to regulate opening and closing of the stomata. Only with a pressure probe (for osmotic pressure determination) (Kappen and Haeger 1991; Franks and Farquhar 2007) or ion probes (and using markers for K^+ transporter molecules) (Raschke and Fellows 1971; Büchsenschütz et al. 2005) is it possible to distinguish S cells (in terms of their mentioned stoma-regulating properties) from non-S cells, some of which can also border the g cells.[4]

The idea that there is a "complex" of cells surrounding a stoma has arisen because observers have tended to focus their attention on the pair of mirror-image guard-cells (g) and have then puzzled over the nature and origin of the surrounding cell(s), some or all of which may be distinctive subsidiary S cells. The guard-cells arise from a guard mother cell, which is produced either directly from m_0 (agenous ontogeny) or from the final m-cell descendent of m_0, following a prescribed number (λ) of divisions in which the m cell is always renewed.[5] For this reason, the guard mother cells are here referred to as m_λ. Zeiger and

[4] All these mentioned studies of S and g cell properties were made on monocot species where the origin of the S cells is different to that of S cells in most dicots. Whether those cells of dicots which are believed to be S cells behave similarly to the S cells of monocots is a question that has been rather neglected by physiologists.

[5] These divisions are often called "amplification" divisions. They are, however, more like a series of asymmetric stem-cell divisions than a series of true amplification, or proliferative, divisions which lead to an increase in the number of cells derived from a stem-cell. They have also been called "formative" divisions by Charlton (1990), a term which we, too, prefer.

Cardemil (1973) have described the timing of meristemoid generation and the subsequent events of stomatal complex development in leaves of *Allium cepa*, but studies with the degree of detail encountered in this citation are rare.

4.2 Cellular Descents

A simple stomatal complex ontogeny is one where all the cells of the complex are descended from a single mother cell, m_0. This type of descent conforms to that of a "*mesogenous*" pathway, or ontogeny, and results in a mesogenous complex (Pant 1965): N, S and g cells all show genealogical descent from cell m_0 and, hence, are "mesogene" cells. By contrast, in a "*perigenous*" ontogeny, all S cells of a stomatal complex are produced independently of m_0 (hence they are "perigene" cells). The m_0 cell divides directly to produce a pair of g cells (i.e. $m_0 = m_\lambda$ and $\lambda = 0$). Then, in a "*meso-perigenous*" ontogeny, some of the S cells of a complex arise independently of the lineage derived from m_0. Hence, they too are perigene cells, whereas other S cells of the same complex are mesogene cells. Whatever their mode of descent, S and g cells co-operate in forming a functional stomatal complex (Sharpe et al. 1987). It is possible, as Galatis and Mitrakos (1979) observed for *Vigna sinensis*, that different ontogenies of the stomatal complex are followed at different times in leaf development (perigenous early on, followed by mesogenous), or when leaves are raised in variable light/dark conditions.

With regard to the development of subsidiary S cells, it could be postulated that, whatever these cells' origin (mesogenous or perigenous), the S-cell state is induced because of the contact of an S precursor cell with an m_0 cell or one of its m descendents. Two events may be required for this induction to be achieved. The first is that, in the case where the S cell is a perigene cell, it should be produced by division of a protodermal P cell which is a neighbour of the meristemoid m_0. This P cell division is often unequal: the smaller daughter adjoins m_0 close to that cell's nucleus and becomes the S cell, its larger sister becomes an epidermal cell (E). It seems that a P cell neighbouring an m_0 cell cannot directly become an S cell, but that it must first divide and thereby produce an S precursor cell. Then, second, there is the induction event, where some or all of the mesogene or perigene cells around an m cell become determined (perhaps due to the influence of the m cell) to differentiate as S cells. In the mesogenous ontogeny, therefore, the larger of the unequally sized descendents of m_0 would be induced towards S state owing to their proximity to the smaller daughter, which retains the meristemoidal state (m). It follows that each and every mesogene S cell descended from an m cell could be derived by induction. Where there is no obvious S cell, there may not be any inductive event whereby cell m_0 (or m_i) confers a new state upon a neighbour cell. Nevertheless, in this agenous pathway or in the mesogenous pathway as it applies to *Arabidopsis* and certain other dicots, the physiological accoutrements of stomatal function are presumably shared between guard-cells and their E neighbours.

4.3 Differences Between Stomatal Complex Ontogenies

Differences between the various stomatal ontogenies listed by Fryns-Claessens and Van Cotthem (1973) may lie, as Timonin (1995) has proposed, in two features of the cellular descent from meristemoid m_0: (1) the number of divisions (λ) of m_0 following its inception from a P-cell up to the division of cell m_λ, which produces the pair of g cells, and (2) the angle θ_a between successive division walls in this sequence of λ divisions. The contributions of each of the variables, λ and θ_a, to the form of the stomatal complex are evident in the diagrams of Farooqui (1981a, b), who has summarised the ontogenies descending from three- and four-sided meristemoids (where $\theta_a = 60°$ or 90°). Another feature used in stomatal classification is (3) the angle, θ_g, of the final division wall (i.e. the angle with respect to the previous division wall in the lineage) by which the pair of guard-cells is formed. Payne (1979), believing θ_g to be important, devised special terms for the respective guard-cell divisions.[6] With respect to the perigenous stomata of barley leaves, Stebbins and colleagues suggested that θ_g was related to the pattern of growth of the guard mother cells in relation to the surrounding cells, and even adduced some indirect experimental evidence for this (Stebbins and Shah 1960; Stebbins 1965a, b; Stebbins et al. 1967; for results from two other grass species, see Palevitz 1982). But the problem of the regulation of θ_g is complex and may require analysis of the microscopic pattern of stresses and strains in the relevant cellular domains to resolve this problem (see the methodologies of Schmundt et al. 1998, for example). Certainly, this division is quite labile (Palevitz and Hepler 1974; Palevitz 1986) and responsive to the growth of the surrounding cells (see Serna and Fenoll (2003) for a brief consideration of this topic).

In the most simple of stomatal ontogenies (the agenous system), $\lambda = 0$.[7] The m_0 meristemoid cell does not undergo any divisions and passes directly to the state of a guard mother cell, m_λ. It then divides to produce two daughter cells, which differentiate as g cells. This is also true of perigenous ontogenies; no divisions intervene between meristemoid m_0 and guard mother cell m_λ. Thus, $m_0 \rightarrow m_{\lambda=0} \rightarrow g'\ g''$. In the other two ontogenetic systems (mesogenous and meso-perigenous), g cells are produced from an m cell, which has undergone at least $\lambda = 1$ division following its

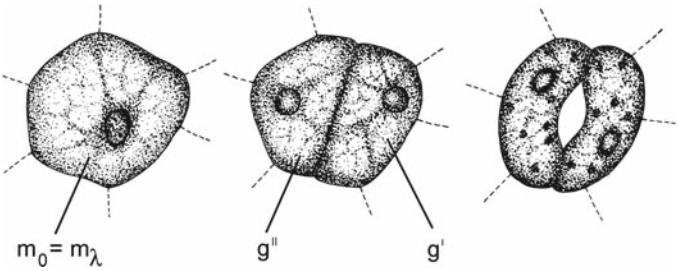
[6] Payne (1979) proposed the terms parameristic, diameristic and anomomeristic for where the respective angles of division of the guard cell, θ_g, were 0°, 90° and irregular (usually 120°). In certain cases, θ_g is regulated by the ability of the division apparatus of the guard mother cell to rotate during mitosis (Palevitz 1986), and this may follow as a consequence of the orientation of leaf surface growth (Lück and Lück 1961).

[7] Agenous stomata have a long history, being found on the surfaces of fossil plants dating from the Silurian and Devonian era (Edwards et al. 1998), thereby pre-dating by millions of years the ancestors of modern plants. It can be speculated that they arose from protodermal cells which occasionally gave rise to guard mother cells, and that these cells, for some reason, failed to expand as much as their neighbouring cells. Maybe their small size was maintained by the development of a special type of cuticle on their external surface. But the small cells could still divide. When they did so, two guard cells were produced. Separation of the division wall between the two sister guard cells brought into being the stomatal pore, which overlies a substomatal chamber in the hypodermis, a chamber produced by a cell separation event that focusses upon the inextensible guard cells above.

descent from m_0. Thus, $m_0 \rightarrow m_1 \ldots m_i$, $m_{i=\lambda} \rightarrow g' g''$. The number of formative divisions has been shown to depend on the size of the meristemoidal daughter cell: when the volume of an m cell is larger than a certain critical value, a further formative division can then occur (Timonin and Cherkashin 1997), and there is also the possibility of additional "satellite" meristemoid formation in one of the extra-large descendents of m (Timonin and Cherkashin 1996). Whether or not this secondary meristemoid divides is also decided by its size – if too large, it will not divide again (Timonin and Cherkashin 1996). Moreover, the value of λ attached to an m cell lineage seems to be related to the intracellular level of some of the various intracellular gene products (e.g. the MUTE-GFP fusion protein, FLP protein, and TMM protein) which regulate meristemoid and stomatal divisions (Nadeau and Sack 2002; Lai et al. 2005; Shpak et al. 2005; MacAlister et al. 2007; Pillitteri et al. 2007), and the expression of the important AGAMOUS-LIKE 16 gene (Kutter et al. 2007).

4.4 *L-Systems and the Production of Stomatal Cellular Complexes*

It is now possible to create developmental systems which formalise the ontogenies of various types of stomatal complex. Four examples are given in Figs. 1–4, together with sketches of the cellular arrangements. The relevant cells and their



$P \rightarrow m_0 \; P_1$ ... A1

$m_0 \rightarrow m_\lambda \rightarrow g' \; g''$

$P_1 \rightarrow S \rightarrow S$

$g' \rightarrow g'$

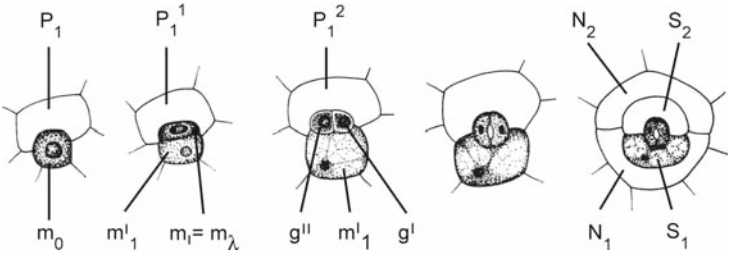
$g'' \rightarrow g''$

θ values do not apply.

Fig. 1 Perigenous or Agenous ontogenetic pathway of stomatal Type No. 1. The Type is numbered according to the system of Fryns-Claessens and Van Cotthem (1973), whose figures have been adapted here. Listed in this Fig. 1 and in the other three schemes of Figs 2, 3 and 4 are the relevant cell state transformations A_1–A_4

states, P, E, m, g, have already been mentioned. Also listed in Figs. 1–4 are the values of λ (number of formative divisions), θ_a (angle between successive division planes), and θ_g (angle between the guard mother cell division and the preceding m cell division).

In Fig. 1 is shown the simple agenous system, which was mentioned in Sect. 4.3. Figure 2 shows a dia-meso-perigenous (or diacytic) stomatal complex. S and N cells lie on either side of the pair of guard-cells; the N cells enclose the S cells, which, in turn, enclose the g cells. Perigene cells S_2 and N_2 are produced by tangential division of cell P_1. This cell, P_1, as well as the mesogene daughter of m_0, cell m'_1, are assumed to be subject to induction by m_0, m_1 or m_λ and, hence, produce S cells when they divide.



$P \rightarrow m_0 \; P_1$ A2

$m_0 \rightarrow m_\lambda \, m'_1$

$m_\lambda \rightarrow g' \; g''$

$m'_1 \rightarrow S_1 \, N_1$

$P_1 \rightarrow P_1{}^1 \rightarrow P_1{}^2 \rightarrow S_2 \, N_2$

$S_1 \rightarrow S_1$

$S_2 \rightarrow S_2$

$N_1 \rightarrow N_1$

$N_2 \rightarrow N_2$

$g' \rightarrow g'$

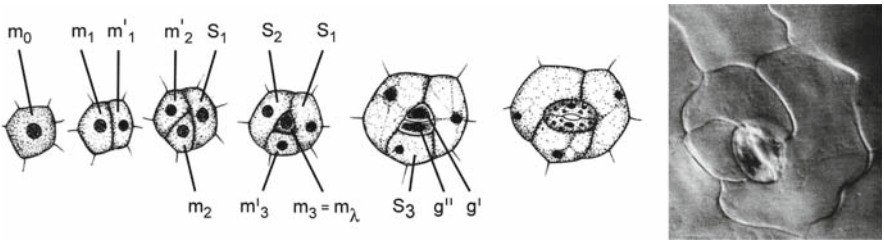
$g'' \rightarrow g''$

$\theta_a = 0^o$, $\theta_g = 90^o$

Fig. 2 Dia-Meso-Perigenous; Type No. 8. The protodermal cell P_1^2 shown above the guard-cells divides and the two daughter cells develop as perigenous S_2 and N_2 cells

When $\theta_a = 60°$, and when different values of λ are operative, either tricytic mesoperigenous ($\lambda = 1–2$) or mesogenous ($\lambda = 3–6$) products arise (Farooqui 1981a). A typical example, which applies to *A. thaliana* (where $\lambda = 3$), is shown in Fig. 3.

Many monocots, e.g. the grasses *Oryza sativa* and *Saccharum* sp., possess a diperigenous stomatal complex (Fig. 4); a tetraperigenous type is found in the Commelinaceae (e.g. *Commelina*, *Tradescantia*). In the last-mentioned case, two P cells proximal and distal to the meristemoid, as well as two P cells on either side of it, are induced to divide unequally and the smaller daughters (which face the m cell) become S cells. The divisions occur in a particular sequence. Cell m_λ then produces the pair of guard-cells.



$P \rightarrow m_0 \quad P_1 \quad \ldots A3$

$m_0 \rightarrow m_1 \; m'_1$

$m_1 \rightarrow m_2 \; m'_2$

$m_2 \rightarrow m_\lambda \; m'_3$

$m_\lambda \rightarrow g' \quad g''$

$m'_1 \rightarrow S_1 \rightarrow S_1$

$m'_2 \rightarrow S_2 \rightarrow S_2$

$m'_3 \rightarrow S_3 \rightarrow S_3$

$P_1 \rightarrow P_1{}^1 \rightarrow E \rightarrow E$

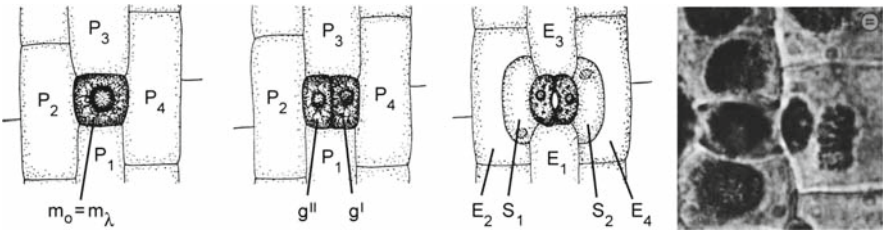
$g' \rightarrow g'$

$g'' \rightarrow g''$

$\lambda = 3$

$\theta_a = 60°, \theta_g = 0°$

Fig. 3 Aniso-Mesogenous; Type No. 24a. Inset micrograph shows a newly formed pair of guard-cells surrounded by three S (or epidermal) cells in *Arabidopsis thaliana* (modified from Serna and Fenoll 2000a)



$P \rightarrow m_0 \quad P_1$ ... A4

$m_0 \rightarrow m_\lambda \rightarrow g' \quad g''$

$P_1 \rightarrow E_1 \rightarrow E_1$

$*P_2 \rightarrow P_2{}^1 \rightarrow S_1 \quad E_2$

$P_3 \rightarrow E_3 \rightarrow E_3$

$*P_4 \rightarrow P_4{}^1 \rightarrow S_2 \quad E_4$

$E_2 \rightarrow E_2$

$E_4 \rightarrow E_4$

$S_1 \rightarrow S_1$

$S_2 \rightarrow S_2$

$g' \rightarrow g'$

$g'' \rightarrow g''$

$\lambda = 0$

$\theta_g = 90^o$

Fig. 4 Di-Perigenous; Type No. 3. In the transformational scheme A4, the two steps marked by an *asterisk* represent a lateral induction event due to cell m_0 acting upon cells P_2 and P_4 which lie at either side. The *inset micrograph* shows a developing stomatal complex of *Hordeum vulgare* (modified from Stebbins 1965a)

Table 1 lists the dwM0L-division systems that produce the various types of stomatal complexes discussed by Fryns-Claessens and Van Cotthem (1973). Here, each division system is based upon an appropriate initial word, ω_0 – i.e. a dextrorse cycle of wall labels that is inherent to the respective meristemoid, m_0 – and a set of cycle state transition rules. The agenous (Fig. 1) and mesogenous ontogenies (Fig. 3), as well as the mesogenous portion of a meso-perigenous pathway (Fig. 2) of dicots, are described in this Table. Perigenous ontogenies of monocots (cf. Fig. 4) have, however, been omitted.

As the entries in Table 1 show, each type of stomatal complex is dependent upon a unique division system for its development. The formal descriptions of the systems may open the possibility of a new classification of stomata on the basis of division system complexity.

5 Meristemoid Site Selection

Deterministic cell productions similar in principle to those which operate during the formation of the stomatal complex, and as described in the Sects. 3 and 4, can be supposed to occur prior to this process, at earlier phases of leaf development (Lück et al. 1988). The location of each meristemoid would, therefore, be the outcome of a division system operating in a younger population of protodermal cells. L-systems have not so far been applied to this problem and, in any case, the protodermal cell lineages at the pre-meristemoidal phase of leaf development, which would allow such an application, are not known.

What feature(s) of a P-cell could influence its division asymmetry? And how can it be that only some of the P-cells are so affected, thereby enabling particular spacing patterns of m_0 cells within the protoderm? In an earlier publication (Barlow and Lück 2008), solutions to these two questions were proposed in relation to the meristemoid which constructs a lateral root primordium on a parent root axis. Here, it was suggested (1) that specific zones of the primary vascular tissue specify the locations (and hence the patterning) of primordia upon the cylinder of pericycle tissue,[8] and (2) that certain older transverse walls positioned at intervals along the pericyclic cell files, together with a younger longitudinal wall that joins this older wall, facilitate the unequal divisions which produce the primordiogenic meristemoids. At least, the second point mentioned might be suggested for stomatogenesis: in many instances, the asymmetric meristemoid-producing division of a leaf protodermal mother cell appears to result, as in the case of the root pericycle cells (Barlow et al. 2004b), from an attraction between that cell's nucleus and one of the transverse walls of the mother cell (see also Hernandez et al. 1999). An attraction between a dividing cell nucleus and a site on a lateral wall also occurs in the case of perigenous S cell productions in monocots (Stebbins and Shah 1960) (see Fig. 4). Moreover, as Stebbins (1966, p. 125) proposed in relation to the initiation of meristemoids in barley leaves, the cells which produce meristemoids are subjected to "a polarity gradient (of hormones), so that all cells undergoing mitosis in the region influenced by them (i.e. the hormones) divide asymmetrically and produce meristemoid cells in the direction from which the hormone is coming". Thus, there is a pre-patterning of the tissue with respect to meristemoid production due to a more global morphogenetic gradient, a feature also remarked upon by Sachs (1974) in relation to his work

[8] Lateral root primordia are arranged with a definite spacing interval in longitudinal ranks along the parent root. The circumferential spacing of these primordia depends upon the distribution of vascular strands. If a root were rolled out flat, in two-dimensions, the distribution of its primordia would resemble that of the meristemoids upon the surface of a leaf.

on stomatal spacing of another monocot, *Crinum americanum*. We may propose that new gradients are then formed, with a source of morphogen in the meristemoid itself. Differentiation of surrounding cells might subsequently occur within the gradient in accordance with a system of morphogenetic thresholds, just as we proposed for vascular cambium and its derivatives (Barlow and Lück 2004b).

As in the pericycle of the root (Barlow et al. 2004b; Barlow and Lück 2008), it might be the age structure of the array of walls of the protodermal cells, which contributes information for both the re-commencement of mitosis and the location of the meristemoid-producing divisions in the protoderm. These walls "fine tune" information present within the larger gradient and direct it to potentially responsive cells. This seems evident in the illustrations of leaf and cotyledon surfaces of *Sinapis alba* prepared by Landré (1972). Unequal divisions and the subsequent generation of m_0 cells occur in the fork of a "Y"-type arrangement of cell walls (Fig. 5), where three cells make contact, the stem of the "Y" making contact with two older walls. Then, after additional divisions, a second generation of m_0 cells along with a new increment of walls are produced "deeper" within the original array; these new m_0 cells lie in proximity to the next-oldest set of "Y" walls. It is as though walls of a certain degree of maturity influence, or initiate, nuclear movement and cytoplasmic polarisation and, thus, determine the inequality of the subsequent cell division. There may also be influences from anticlinal walls of cells in the sub-protodermal layer, as suggested by Serna et al. (2002).

Because of the linearity of the cell files of monocot leaves, it is possible to examine the siting of meristemoids in ways that take advantage of this geometrical feature. For example, it has been suggested that meristemoid formation is related to position in the cell cycle of the precursor cell as it is displaced away from the basal meristematic zone of the leaf (Charlton 1990). Chin et al. (1995) were able to

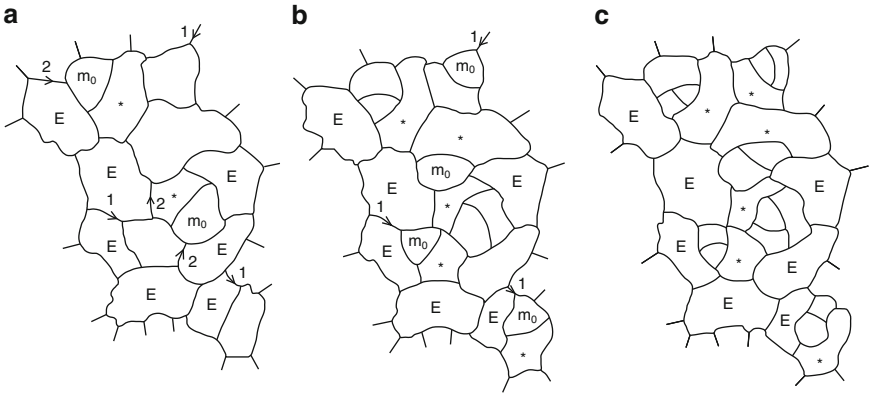


Fig. 5 Development of a succession of m_0 cells in the cotyledonary protoderm of *Sinapis alba*. (**a**) Division wall 2 joins with an older wall making a 'Y' junction; (**b**, **c**) new division walls of the next cell generation are marked 1. The number against each wall is retained for two generations. Unequal divisions occur as though the nuclei of the mother cells had been drawn (by the *arrows* on walls 1 or 2) towards suitably aged 'Y' junctions prior to mitosis. Asterisk – sister cell of m_0; E – epidermal cell. Reconstructed from Fig. 24 of Landré (1972)

adduce evidence in support of this proposal from observations on the distribution of nuclear DNA contents along the files of protodermal cells of *Tradescantia* leaves. Thus, relationships between cell cycling and cell size in an environment that supports stomatogenesis may also have some bearing on the pattern of meristemoid formation. If this is so, then the natural variation in cell sizes generated by a determined number of cell cycles (λ) and division asymmetry within a file of cells (Lück et al. 1994) may assist in determining meristemoidal sites (Barlow and Lück 2008), not only in monocots but also in dicots.

Stomatal spacing within the abaxial and adaxial epidermal cell layers (Croxdale 2000; Bergmann 2004) is another problem to be solved. The developing leaf vascular system (veins and veinlets) within the sub-epidermal space may influence the location of stomata. In this respect, spacing might be connected with the lineages of the cells, which are revealed by the so-called "areoles"[9] of leaf surfaces (Terashima 1992). In both monocots and dicots, the presence of nearby veins is said to deny stomatogenesis in the overlying protodermal cell layer (Sachs 1974; Wenzel et al. 1997; Serna and Fenoll 2000a), but there can be exceptions (e.g. Galatis and Mitrakos 1980 – see their Fig. 37). In grass leaves, the exclusion of stomata from protoderm over a vein may be due to that tissue's cap of sclerenchyma.[10] This speculation is supported by findings from leaves of the *Xcl1* mutant of maize (Kessler et al. 2002): stomata on the leaf surfaces were reduced in frequency, suggesting that the extra layers of cells, which form under the protoderm of this mutant, prevents morphogen from reaching the protoderm and thereby denies the unequal divisions needed for meristemoid production. One candidate morphogen that might assist the regulation of meristemoid spacing is auxin (Barlow et al. 2004b). Thus, an auxin, such as indole acetic acid may be the inducer of unequal divisions in the protoderm as it moves from the developing veins into each areolar domain.

The rates and durations of stomatogenesis during leaf growth are quite diverse amongst species (Carr and Carr 1990) and the factors regulating them are little understood. Differences between stomatal numbers and densities upon the upper

[9]The areole of the leaf is not to be confused with the quite different areole of cactus stems! The leaf areole domain referred to here has also been designated by the more cumbersome, but more exact, term "bundle sheath extension compartment" (Terashima 1992). "Stomatal complex domain" or "stomatal complex compartment" might be equally appropriate terms. The areole comes to develop as a packet of related cells which contains a generative cell (meristemoid) with regular cell productions, just as postulated for primitive apices (Barlow et al. 2000). Then, one set of cell walls (say, the oldest walls) bordering the areole cell-packet initiates vein formation. Vein formation itself can also be considered as a deterministic process (Lück and Lück 1981). In this respect, it could underpin some of the other, but equally hypothetical, chemical morphogen-based scenarios for leaf vein development. The formation of veins and areoles is also reminiscent of the cracking of surfaces due to their expansion or contraction (Iben and O'Brien 2009). Thus, veins might follow the path of intercellular 'cracking', or splitting, and the areas of tissue (especially epidermis) thus defined become areoles within which stomatal meristemoids form.

[10]Despite extensive quantification of features of the leaf epidermis in relation to stomata (e.g. Tichá 1982), relatively little is known about the spatial disposition of cell types immediately below the epidermis and its structures.

and lower leaf surfaces may reflect the respective times of initiation and conclusion of the stomatogenic process within certain "windows" of plastochronic development (Carr and Carr 1990). But differences in stomatal index (fraction of all epidermal cells which are stomata), as well as the different ratios between the size of guard-cells and their pores, may be due to other types of control, such as the relative amount of auxin which passes through young abaxial and adaxial leaf surfaces (Stebbins 1966; Sachs 1991). In view of the importance of stomatal density in the water economy of the whole plant (Wang et al. 2007), it is of some practical importance to understand this feature of plant development. The *ERECTA* gene of *Arabidopsis* has provided a starting point for such research (Masle et al. 2005) and has led to the discovery of epidermal patterning factor 1 (*EPF-1*) gene (Hara et al. 2007). Another possible regulatory control of stomatal density is the wax composition of the leaf surface (Bergmann 2004; Casson and Gray 2008).

6 Satellite Stomatal Complex Formation

There are two aspects to the production of new m_0 cells. The *first* refers to what may be called a "generational increase": new m_0 cells continue to be produced within the increasing population of P cells; and, as already proposed in Sect. 5, m_0 cell production may be related to the production of suitably aged wall junctions against which asymmetric divisions can occur. The timing of successive "waves" of stomatogenesis have been examined for developing leaves of both *S. alba* (Landré 1972) and *Eucalyptus* spp. (Carr and Carr 1990).

A *second* type of increase of m_0 cell production flows directly from certain of the division systems outlined in Table 1. That is, mother cells for a new generation of meristemoids m_0 arise within and amongst the descendents of earlier m_0 cells according to a deterministic plan. This mode of increase was first noticed in leaves of Brassicaceae (Paliwal 1967) and Caryophyllaceae (Inamdar 1968), and has also been found in leaves of *Pisum sativum* (Kagan et al. 1992) and *A. thaliana* (Larkin et al. 1997; Serna and Fenoll 2000a). The time-course of stomatogenesis in *A. thaliana* seems to be linear: production of guard mother cells (m_λ) on the adaxial leaf surface ceases after a total of about 1,000 epidermal cells have been formed, whereas on the abaxial leaf surface m_λ production continues up to a stage where there are about 2,000–3,000 epidermal cells (Geisler and Sack 2002). Many of these extra satellite stomata form from second- and higher-order "satellite" m_0 meristemoids. Branching of the stomatal cell lineage should not come as a surprise. An analogous branching occurs in meristems of lower plants and involves the autoreproduction of apical cells, which have the potential to develop new organs. In this respect, stomatal meristemoids seem similar in behaviour to the apical cells of shoots and roots, except for the number of proliferative divisions which follow after the prescribed number, λ, of formative divisions have ceased. dwM0L-system algorithms describe the relevant development pathways of apical cells quite exactly (Barlow et al. 2001; Barlow and Lück 2004a), as they do for stomatal meristemoids (Table 1). Somewhat ironically, in *Psilotum nudum*, the very species in which

auto-reproductive cellular behaviour within its apical meristems was studied in detail (Barlow et al. 2000, 2001; Barlow and Lück 2004a), the stomatal meristemoids do not show such auto-reproductive behaviour (Barlow and Lück 2007). Here, stomatogenesis is of the agenous type (Fig. 1): when the *Psilotum* meristemoid divides, it produces directly a pair of guard-cells (i.e. $\lambda = 0$) (Pant and Khare 1971).

A strict pattern of satellite m_0 productions prevails in *A. thaliana* whose stomatogenesis follows the aniso-mesogenous Type 24 of Fryns-Claessens and Van Cotthem 1973 (see Fig. 3). A value of $\lambda = 3$ applies to the cell lineage of m_0, and $\theta_a = 60°$. As a result, three cells (S or E) surround the guard mother cell ($m_{\lambda=3}$). The division of the guard mother cell is anomomeristic with $\theta_g = 90°$. The stomatal complex adheres to a "one-cell rule" (Sachs 1991; Serna and Fenoll 2000b; Hara et al. 2007) that precludes stomata touching each other; but in this case, the one-cell rule is itself the outcome of a purely deterministic process that follows from a particular division rule.

The repetitive formation of m_1 m'_1 (ancestors of the guard-cells g' and g'') is shown in Table 2 and in the following state transition derivation, A5:

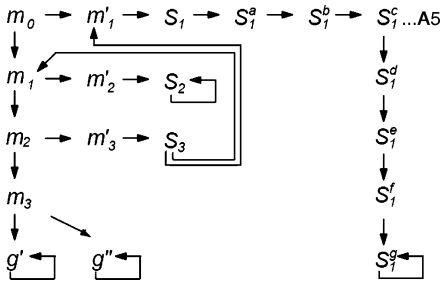


Table 2 Division and transformatory systems for the development of satellite stomata in *Arabidopsis thaliana*. The systems relate both to the cells and to the constituent wall elements. Representation of the basic division system is found in Table 1 at ontogenetic Type 24b, and in Fig. 3

Cell labels	Wall segment cycles	Cell transformation rules	Wall transformation rules
m_0 (like S_3)	(1 2 3 4 5)	$m_0 \rightarrow m_1\ m'_1$	(1 2 3 4 5) → (4 5 6/$_1$2 3/$_1$2 3)
m_1	(1 2 3)	$m_1 \rightarrow m_2\ m'_2$	(1 2 3) → (4/$_1$2 3/$_1$2 3)
m_2	(1 2 3)	$m_2 \rightarrow m_3\ m'_3$	(1 2 3) → (4/$_1$2 3/$_1$2 3)
$m_{\lambda=3}$	(1 2 3)	$m_\lambda \rightarrow g'\ g''$	(1 2 3) → (c/$_a$b c/$_a$ b)
g'	(a b c)	$g' \rightarrow g'$	(a b c) → (a b c) = final
g''	(a b c)	$g'' \rightarrow g''$	(a b c) → (a b c) = final
m'_1	(1 2 3 4 5 6)	$m'_1 \rightarrow S_1$	(1 2 3 4 5 6) → (12 3 4 5 6 7)
m'_2	(1 2 3 4)	$m'_2 \rightarrow S_2$	(1 2 3 4) → (12 3 4 5)
m'_3	(1 2 3 4)	$m'_3 \rightarrow S_3$	(1 2 3 4) → (12 3 4 5)
S_1	(1 2 3 4 5 6 7)	$S_1 \rightarrow S_1{}^a$	(1 2 3 4 5 6 7) → (12 3 4 5 6 7 8)
$S_1{}^a$	(1 2 3 4 5 6 7 8)	$S_1{}^a \rightarrow S_1{}^b$	$S_1{}^a = S_1{}^b$
$S_1{}^b$	(1 2 3 4 5 6 7 8)	$S_1{}^b \rightarrow S_1{}^c$	(1 . . . 8) → (12 3 4 5 6 7 8 9)
$S_1{}^c$	1 . . . 9)	$S_1{}^c \rightarrow S_1{}^d$	(1 . . . 9) → (12 3 4 5 6 7 8 9 0)
$S_1{}^d$	(1 . . . 0)	$= S_1{}^e = S_1{}^f$	
$S_1{}^f$	(1 . . . 0)	$S_1{}^f \rightarrow S_1{}^g$	(1 . . . 0) → (1 23 4 5 6 7 8 9 0 A)
$S_1{}^g$	(1 . . . A)	$S_1{}^g \rightarrow S_1{}^g$	(1 2 3 4 5 6 7 8 9 0 A) = final
S_2	(1 2 3 4 5)	$S_2 \rightarrow S_2$	(1 2 3 4 5) → (1 2 3 4 5) = final
S_3	(1 2 3 4 5)	$S_3 \rightarrow m_1\ m'_1$	(1 2 3 4 5) → (4 5 6/$_1$2 3/$_1$2 3)

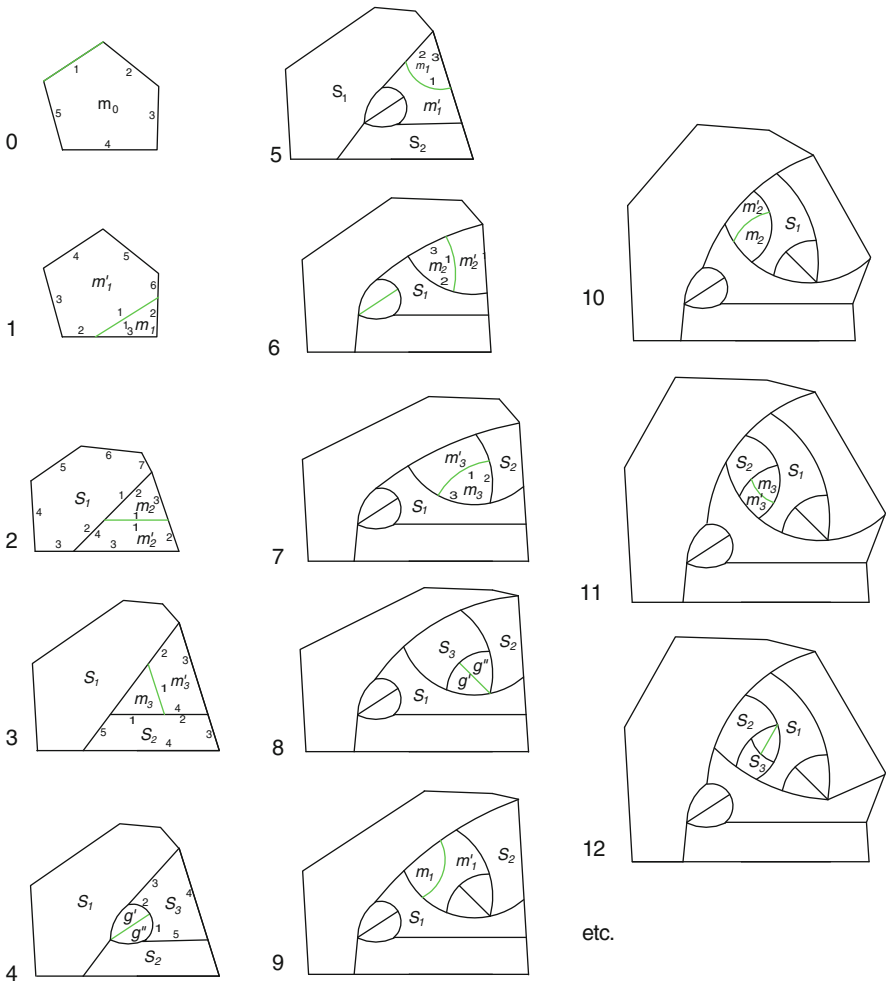


Fig. 6 Development of satellite stomata in the protoderm of *Arabidopsis thaliana* in accordance with the division system given in A5 and Table 2. Division sequences are based on those illustrated by Larkin et al. (1997) except that here the meristemoids and stomata are produced in a sinistrorse spiral instead of the dextrorse sequence shown in that publication. A five-sided cell is taken as the starting point. New division walls are denoted by green lines

The number of labels in each wall cycle (Table 2) indicates the number of walls belonging to each cell. A map of the corresponding cell and wall productions is given in Fig. 6. Cell S_1 enlarges and makes contact with more neighbour cells, whereas cell S_2 remains small and has fewer wall segments and contacts. Cell S_3 recycles indefinitely thus initiating a succession of new satellite stomata. The positions of the satellite stomata are determined in accordance with the state transformations of the walls. We therefore suggest that a deterministic state transition rule, such as evidenced in Table 2 and Fig. 6, is a sufficient condition for the

production of satellites. Also, it would "explain" the so-called "one-cell-spacing rule" (Von Groll and Altmann 2001; Serna and Fenoll 2000b; Serna et al. 2002; Hara et al. 2007). The spacing is in fact the predicted outcome of an epigenetically based rule which specifies the timing and the positioning of new division walls. The requisite wall-state transformations have their cytological counterpart in the wall and underlying cytoplasm, as proposed earlier (Barlow et al. 2000) for shoot apical cell divisions. No doubt, genetic controls, such as shown by the *EPF-1* gene (Hara et al. 2007) are important, but controls, such as these feed into an already formed epigenetic system. This system may itself be due to a physiological definition of the initial word ω_0 within the meristemoid m_0 as a result of earlier-acting genes.

The numbers of first-, second- and third-order satellite stomata of *Arabidopsis* are increased by mutation at the *STOMATAL DENSITY AND DISTRIBUTION-1* (*SDD-1*) locus (Berger and Altmann 2000). As a consequence, the total number of stomata per leaf increases 2-fold and the stomatal index increases 3–4-fold. Possibly, these increases are due to a prolongation of the time during which stomatogenic cell productions take place. And, as shown by Kutter et al. (2007), regulation of satellite stomata number can come from alterations in the expression of a gene such as AGL-16, or the rate at which its products are degraded. The question arises concerning how many other division systems would produce satellite meristemoids if the period of growth and division of the initial cell were extended (as may happen in the mutation *peapod* of *A. thaliana* (White 2006)).

7 Mutations and Stomatogenesis

Mutations have been induced in *A. thaliana* which affect three key stages of stomatogenesis: (1) the unequal "entry" cell division of protodermal cells and the production of meristemoids, m_0; (2) the completion of λ formative (or amplification) divisions; (3) the production of two guard-cells. Not only do the mutations map the pathway, or transformation, of gene activity required for stomatogenesis, but also they allow identification of genes uniquely linked to leaf stomatal complex development, thus distinguishing them from genes that regulate asymmetric divisions which occur elsewhere within the plant (see Scheres and Benfey 1999). The products of stomatal-regulating genes in *Arabidopsis* bring insights to the cytological underpinnings not only of that species' stomatal complex division system, but also, perhaps, of plant stomata in general.

The mutation *four lips* (*flp-1*) was one of the first to be described as affecting stomatal complexes in *Arabidopsis* (Yang and Sack 1995). The stomata of *flp-1* are paired laterally as a result of each guard mother cell undergoing two longitudinal divisions. Although such extra divisions are normally absent from *Arabidopsis*, extra guard mother cell divisions sporadically occur in some other species (Farooqui 1979) and produce the same phenotype as *flp-1* (Rao and Ramayya 1967). Examination of DNA from 17 other species of monocots and dicots has shown sequences similar to the *FLP* gene in all of them (Lai et al. 2005).

Occasionally the guard-cell will subdivide and generate an extra stoma (Farooqui 1979). Guard-cell transverse division can also be induced when plants are grown in abnormal atmospheric environments (Kazama et al. 2004).

The cell state transition rule, $m_\lambda \rightarrow v_g \rightarrow g'\, g''$, could be inserted into algorithm A3 (which applies to *Arabidopsis* stomatogenesis) in anticipation of some event affecting the second of the two transformations ($v_g \rightarrow g'\, g''$), as appears to be the case in the *flp* mutation. The λ value of v_g (λ_{vg}) would normally be 1, but in *flp-1* $\lambda_{vg} = 2$. In the mutation *flp-7*, however, $\lambda_{vg} = 3$, which leads to chains of paired guard-cells on account of two extra longitudinal cell productions. In the double mutant of *flp-7* and *myb88*, $\lambda_{vg} = 4$, thus increasing the number of stomatal cells in the cluster up 16 or more. The percentage of stomatal clusters per unit area of abaxial leaf surface increases from 0 in wild-type *FLP*, to 20, 40 and 80 in *flp-1*, *flp*-7 and *flp-7-myb88*, respectively (Lai et al. 2005). An interesting question addressed by Yang and Sack (1995) is whether mirror-image pairs of guard-cells, as well as the stomatal pore separating them, arise from the extra divisions of a stem-cell-like guard mother cell or from its daughters. Groups of guard-cells with uneven numbers of cells were often seen, especially in the double mutant. The origin of the "odd" cells is unclear, but they probably arise by division of just one of the pair of daughter cells.

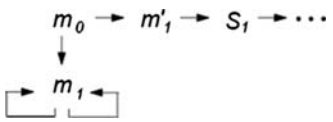
Another significant mutation is *too many mouths* (*tmm*) (Yang and Sack 1995; Nadeau and Sack 2002; Serna and Fenoll 2002). It reveals something about the production of satellite stomata in *Arabidopsis*. The *tmm* mutant has three defects: (1) a cell adjacent to v_g or g divides unequally to give a satellite mother cell (a secondary meristemoid m_0); (2) the sister cell of a satellite m_0 cell becomes a v_g cell and then produces a pair of guard-cells, g' and g''; (3) satellite m_0 cells divide less frequently than normal, resulting in a decreased number of cells between stomata and, hence, in an increased probability that pairs of stomata will contact each other (Geisler et al. 2000, 2003). It seems that, in *tmm*, the daughter cell of m_0 divides unequally with an orientation that is more random than in the wild-type, where division orientation is more strictly controlled; the smaller daughter of this second unequal division becomes a guard mother cell and produces the extra stoma. Whereas in the small neighbouring cell of m_0 and its m descendents the nucleus circumambulates the cytoplasmic periphery, in larger and older neighbouring cells the nucleus comes to lie in a distal location with respect to the m cell (Geisler et al. 2003). It may be that the information resident in walls or peripheral cytoplasm which normally governs the insertion of division walls (information for which is encoded in the wall-state transformation rules shown in Table 2) is abolished. Wall insertion consequently falls solely under the influence of nuclear position: wherever the circumambulating nucleus happens to be located when it is about to divide, there a new division wall will form.

The discovery of three mutations, *speechless* (*spch*), *mute* and *fama* (see Pilletteri and Torii 2007; Pillitteri et al. 2007; MacAlister et al. 2007; and see minireviews by Barton 2007; Gray 2007; Martin and Glover 2007; Serna 2007; ten Hove and Heidstra 2008; Nadeau 2009) has also been significant for stomatogenesis research. The mutant *spch* cannot produce m_0 meristemoids and, hence, no stomata form. Over-expression of *SPCH* results in unequal divisions in all protoderm cells

(MacAlister et al. 2007) and the overproduction of meristemoids and stomatal guard-cells. *MUTE* terminates formative divisions in the descendents of m_0; i.e. *MUTE* sets the value of λ. *mute* meristemoids undergo extra unequal divisions without finally producing a pair of guard-cells. The result is a rosette of cells of decreasing size, the angle (θ_a) between successive divisions having been maintained, as usual, at about 60°. Expression of *FAMA* is restricted to the guard-cells. *fama* mutants produce side-by-side chains of cells in place of the normal single pair of such cells (the phenotype of *fama* is similar to *flp*). *FAMA* is, therefore, thought to restrict guard mother cell divisions and to promote guard-cell determination; it may function in concert with *FLP* and *MYB88* genes, though on a different part of the stomatogenic pathway (Ohashi-Ito and Bergmann 2006). Over-expression of FAMA results in each protodermal cell forming a single, unpaired guard-cell.

To formalise the stomatal cellular productions and state transformations in *mute*, algorithm A5 has to be modified slightly. It is the step $m_1 \rightarrow m_2\ m'_2$ which is affected.

As shown in the derivation graph below, which applies to *mute*, cell m_1, once it has been produced, continues to reproduce itself. Hence:



8 Clustered Stomatal Complexes

Clusters of stomatal complexes occur naturally on the leaf surfaces of some species: they are well known in *Begonia* spp, for example (Boghdan and Barkley 1972). After considering the geographical distribution of two species of *Begonia* (*B. heracleifolia* and *B. nelumbiifolia*), Hoover (1986) suggested that the stomatal clusters assist water conservation of those plants growing in dry soils. Experimental evidence from *Cinnamomum camphora* (Zhao et al. 2006a), which also exhibits stomatal clusters, supports this idea. This species showed a negative correlation between the density of clusters and soil moisture. The density of stomata showed no such correlation, however.

The origin of clusters in *B. peltatifolia* can be deduced (Fig. 7) from the illustrations shown by Tang et al. (2002). One way of producing a cluster seems to be for an m_0 cell to divide twice and hence produce a tetrad of m_0 cells. Each of these four cells then participates in a helicoidal sequence of divisions. In each stomatal complex within a cluster $\lambda = 4$ and $\theta_a = 120°$. The final division in each developing complex produces a pair of guard-cells. The individual stomatal complexes composing the clusters correspond to the helico-mesogenous Type 25 of Fryns-Claessens and Van Cotthem (1973). Are the four cells of the aforementioned

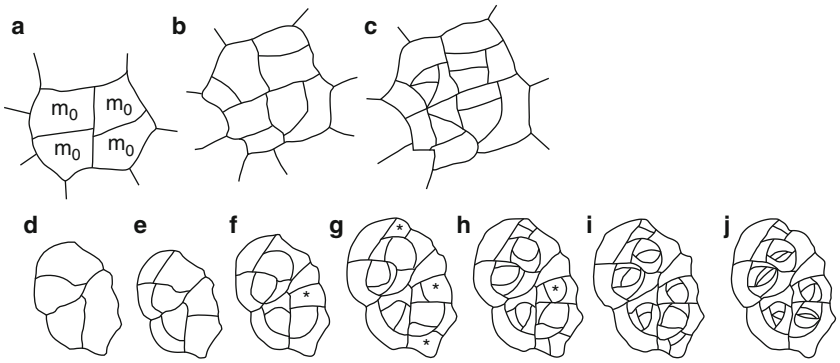


Fig. 7 Clustered stomatal complex formation in *Begonia peltatifolia*. (**a**) A quartet of equally sized, second-order m_0 cells produced from an earlier, first-order m_0 cell derived by unequal division of a protodermal cell. (**b**, **c**) Reconstructed sequence of divisions in another quartet of small cells which are now on the pathway to stomatogenesis. (Redrawn (**a**) from Fig. 3, and (**b**, **c**) from Fig. 4 of Tang et al. 2002). (**d**)–(**j**) Reconstructed sequence through six steps of cluster formation. Extra divisions occur in cells marked by *asterisk* in (**f**), (**g**) and (**h**) and may initiate satellite stomata. The unequal division in the lower right quadrant of the quartet in (**e**) probably leads to the nearly complete satellite stoma evident in (**j**). (Redrawn from Fig. 9 of Tang et al. 2002)

tetrad meristemoids? If so, why do they not inhibit each other's development, as might be expected if Bünning's view of the meristemoid is correct (Bünning 1956)? Maybe here is another subject for investigation.

The fact that, in young leaves of *B. peltatifolia*, clusters of four stomata are the most frequent of all cellular configurations (Tang et al. 2002). This suggests that quartets of stomatogenic cells originating from the first m_0 cells may be the norm for this species. Similar ontogenies for stomatal clusters can be deduced for other *Begonia* spp. from illustrations of Bünning and Sagromsky (1948). It seems that there is even mutual attraction between the nuclei of the four cells involved in the subsequent asymmetric divisions (see Bünning and Sagromsky 1948, Fig. 20). The pathway for clusters in *B. peltatifolia* continues with the production of satellite meristemoids which form within certain derivatives of the original m_0 cell (Tang et al. 2002). These secondary and higher-order meristemoids probably account for clusters with up to 8, 9 or even 13 stomatal complexes. Low levels of soil moisture, which favour stomatal clustering, would therefore seem to promote these satellite cell divisions, possibly on account of a changed relationship between the rates of cell enlargement and cell division.

Zhao et al. (2006b), who studied the abaxial surfaces of leaves of *C. camphora*, illustrates a group of three stomata – one large, older one and two small, younger ones – within an areole which had been produced from a single protoderm cell (Fig. 8). The two smaller stomata seem to be secondary stomata within a group of cells of common descent (the areole). Direct stomatal contact is said to be rare in this material (Zhao et al. 2006b), so any cellular pattern where such contact occurs

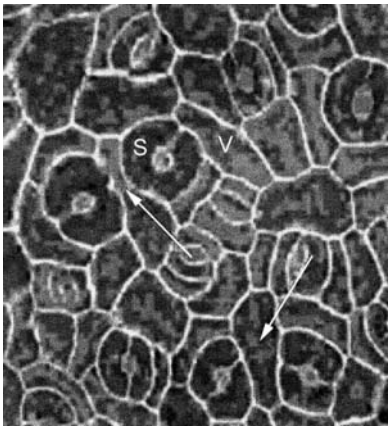


Fig. 8 Photomicrograph of stomatal complexes on a leaf of *Cinnamomum camphora*. The cells are confined within a putative areole (defined by the boundary of a cell cluster) where cell v overlies a veinlet. The large stoma and guard-cells, s, were the first to be produced in the descent from a meristemoid, the two smaller stomata to their right arose later. The *arrows* indicate cells, such as v, which separate one pair of guard-cells from another pair. Modified from Fig. 2a of Zhao et al. (2006b)

is likely to have been excluded during the evolution of the division system which produces this species' stomatal complex. The dwM0L-system which reproduces this ontogeny is given in A6. The corresponding map is shown in Fig. 9.

$(1\ 2\ 3\ 4) \rightarrow \left(4/_5 6\ 7\ 8/_1 2\ 3\right)$ for all steps except $3 \rightarrow 4$,

$(1\ 2\ 3\ 4) \rightarrow \left(8/_1 2\ 3\ 4/_5 6\ 7\right)$ for step $3 \rightarrow 4$ (mirror image)

i.e. successively $\lambda = 4$, $\lambda = 3$, and $\lambda = 1$.

$(5\ 6\ 7\ 8) \rightarrow \left(4/_5 6\ 7\ 8/_1 2\ 3\right)$ for step $3 \rightarrow 4$,

$(5\ 6\ 7\ 8) \rightarrow \left(7\ 8/_1 2\ 3\ 4/_5 6\right)$ for step $5 \rightarrow 6$ (mirror image) ... A6

The cell productions from A6 (and shown in Fig. 9) illustrate two interesting features which, perhaps, could not be fully appreciated except by approaching cell patterning by means of dwM0L-systems. First, there is a defined sequence of cell divisions that could not be guessed intuitively. Second, the cellular map displays a result analogous to that expected from the application of a putative physiological one cell rule. The "one cell" which intervenes between stomata, precluding their contact, is positioned in accordance with the deterministic division system using the mirror image inversion step supplied by A6. Without this step, the final stoma would be placed at right angles to the previous one and would lack a one cell space.

Sometimes, pairs of stomata are regularly found, as in the leaves of Liliales (Rasmussen 1983). It is possible that a division of a protodermal cell, which already contains stomatal determinants, occurs before any meristemoid becomes evident, and that, following its division, two of the daughter cells each produce an m_0

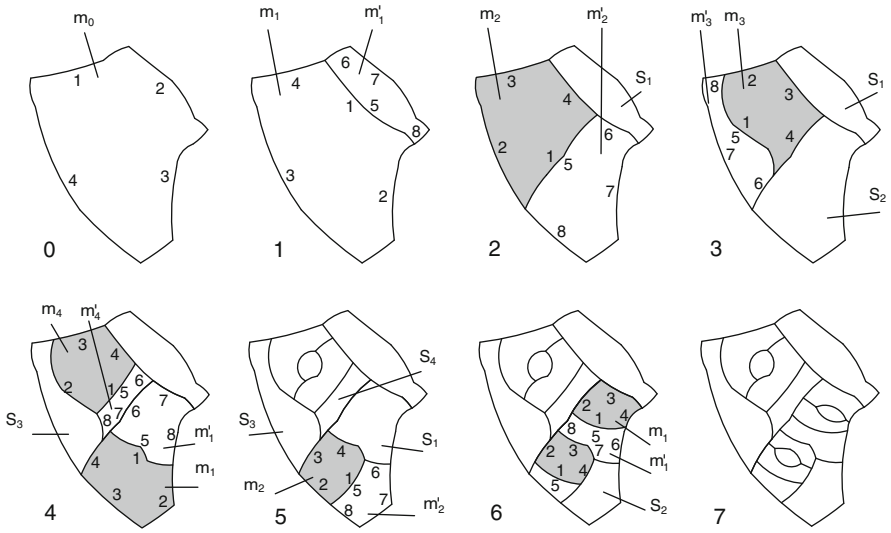


Fig. 9 Map simulating the development of a stomatal complex of *Cinnamomum camphora* (cf. Fig. 8) by means of a dwM0L-system whose rules are given in algorithm A6

meristemoid at the next division. This also could be the means by which the characteristic paired stomata in needles of *Pinus* species are produced (Johnson and Riding 1981).

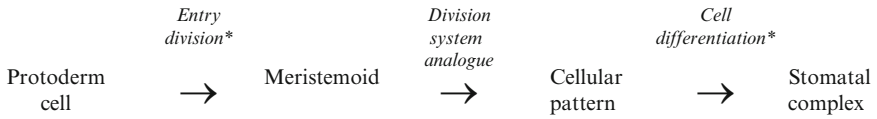
9 Cellular Relationships Associated with Stomatogenesis

How is it that nature has permitted such extraordinary diversity of arrangements of cells around the stomata of leaves? Twenty seven ontogenetic types were proposed for angiosperms by Fryns-Claessens and Van Cotthem (1973), and 24 types, many of them not represented in the angiopserms, were reported for ferns by Sen and De (1992). More recently, 220 different stomatal cellular patterns have been collected from 500 species of angiosperms (Prabhakar 2004), and these have enabled a topological classification of stomatal complexes (J Lück in preparation).

Diversity of cellular patterns within and between species probably results from (a) the distinctive shapes, sizes and interactions between protodermal cells, (b) the distribution of the developing system of veins within the young leaf and, hence, (c) the division of the leaf surface into areolar compartments (see also footnote 9 on page 20). Cellular patterning of stomatal complexes amongst the basal angiosperms seems to involve noticeable amounts of tangential division in the cells neighbouring the guard-cells (Carpenter 2005). Such divisions may be responses to stress or strain patterns within the expanding leaf surface. The tough, inextensible guard-cells or their precursors (see footnote 7, p. 13) could be located within

the protoderm where cells are subjected to physical–mechanical forces which induce their tangential division (see Lintilhac and Vesecky 1984). If so, then such forces must have been rectified over evolutionary time because tangential divisions are not a great feature in the majority of stomatal complexes (see Table 1, and also Fryns-Claessens and Van Cotthem 1973). Interestingly, however, in *Vigna sinensis* they can be seen preferentially in complexes which lie over veins (Galatis and Mitrakos 1980), suggesting that these tangential divisions might be stress-related responses.

The principal that intra-tissue physical forces influence the planes of cell division may also clarify some of the perigene cell patterns and the means of their induction. For example, in leaves of *Gingko biloba*, pairs of guard-cells differentiate before the surrounding cellular pattern of the stomatal complex is established (Kausik 1974). Only later, when stomata become functional and with opened pores, do tangential divisions occur in neighbouring protoderm (P) cells; pressure caused by the opening of the guard-cells may induce these divisions. However, whether the S cells which are suggested to have been created (according to Kausik 1974) by these divisions are actually different from the surrounding epidermal pavement cells is not known. Nevertheless, the example alerts us to the possibility that a physico-mechanical effect may be a sufficient condition to initiate a developmental feature, which otherwise might be proposed as being mediated by the cell-to-cell passage of a chemical morphogen. However, we do not wish to give the impression of belittling cell physiology in the context of stomatal complex formation: it is almost certainly through gene-mediated physiology that both the location of the meristemoid m_0 and for its initial word ω_0 are defined, and the same might be true also for the number of divisions, λ, of m_0 and for the types of cells that differentiate after their production from the meristemoid. In the scheme below, the starred processes (*) involve gene action:



The ontogenetic pathways leading to the various stomatal complexes (cf Table 1) are another matter. They may result from deeper, epigenetic and genetic processes, as well as from physico-mechanical effects inherent to the superficial cell layers of leaves. It may be that the striking differences between the periginous and mesogenous pathways have their origins in different modes of leaf growth: linear and basifugal growth in the case of the leaves of grasses, for example, in contrast to the more isotropic growth of the leaves of dicots. Many of the stomatal complex patterns are perhaps outcomes of ever-present teleological “searches” that operate every time an ontogenic pathway is embarked upon; and this occurs time and again

in successive generations of plants. The "aim" of such pathways is, after all, the production of an optimum system of physiological and structural support for the guard-cells. A further system – the "sieve of evolution" – then selects and fixes, and thereby upgrades, each pathway. Some of the established ontogenetic pathways are generated by meristemoids (the mesogenous pathways), others (the perigenous pathways) make use of already formed groups of cells.

An early study of stomata of just a few species of monocots lent credence to the idea of there being on-going "searches" for optimum stomatal systems (Stebbins and Jain 1960). The cellular patterns considered at that time ranged from the simple stomatal complex of *Allium cepa* with perhaps one or no S cells (see Fridvalsky 1957), to *Hordeum vulgare* and *Rhoeo discolor* with two and four S cells, respectively, and then to *Commelina communis* with six S cells. Interestingly, a similar ontogenetic progression, again recognised as an amplification of the number of S and N cells, was suggested from a survey of over 200 species of ferns by Sen and De (1992). One interpretation of the variation in cell numbers contributing to stomatal function is that there has been a tendency towards constructional complexity, with more and more S cells being added to the stomatal system during phylogeny, presumably in order to enhance stomatal "efficiency". However, observations from another, larger, survey of monocots were believed by Stebbins and Khush (1961) to indicate that the reverse was the case: that stomatal complexes had become simplified during phylogeny. In this context, it is interesting to note that, in dwM0L-systems, a consideration of the size of the alphabet, ω_0, shows that morphologically simple structures can actually be more complex in terms of the number of wall labels required to bring about their construction than more complicated structures, which can be constructed with fewer labels (Lück and Lück 1985). The question comes down to how complexity of a structure is defined.

Further questions are: what alterations in the biotic (internal) and abiotic (external) environments could have favoured ontogenetic transformations which diminish (or increase) the number of constructional events for a stomatal complex? Or could it be that phylogeny does not follow any goal-directed progression based on natural selection, and that ontogenetic variation is simply the result of the "fixing" of constructional alterations to developmental pathways which have no particular adaptive advantage or disadvantage? Generally speaking, one particular pathway, or ontogenetic type, rather than a range of types, is characteristic of a given species; that is, certain pathways of stomatal complex ontogenesis do become fixed, at least those utilised during the development of leaf surfaces. Snir and Sachs (2002), however, present an alternative view. They demonstrate that many variations in division pattern can occur withing a species. These variations, nevertheless, are characteristic of the species.

All mesogenous and meso-perigenous pathways arise as variants on a theme commencing with the basic unit of a single meristemoidal cell. Then, after a "decision" that additional cells are needed to develop the stomatal complex, there is the deployment of different numbers of formative divisions (with different λ values) and angles of cell division (θ_a, θ_g). Variations of θ_a are also the basis of the three- or four-sided apical cells at the summits of apical meristems of plant

organs (see Barlow 1994). Apical cells are analogous to stomatal meristemoids[11] but differ from them by the possession of greater numbers of stereotypic formative divisions which give way to proliferative (amplification) divisions. One possible factor in the natural selection of apical cells with particular numbers of wall facets, and hence with particular values of θ_a associated with their formative divisions, is that a certain energy cost is involved in moving a nucleus within the apical cell so that it can become oriented correctly for a forthcoming division (Barlow 1994). However, an excessive energy cost accumulated by a mesogenous stomatal ontogeny could be relieved by the adoption of an agenous or a perigenous ontogeny. This might have occurred when a dicotyledonous (mesogenous) line made the transformation to a monocotyledonous (perigenous) line. Alternatively, there may be the opportunity to develop some of the complexes by means of self-assembly: for example, the aniso-mesogenous complex of *A. thaliana* can be developed (J Dumais, personal communication) by application of Errera's rule that equal cell division should be accomplished by a partition wall of minimal area.

Just as the basis for the mesogenous ontogenetic pathway lies in the informational states of the cell wall and cortical cytoplasm of the meristemoids and their descendents, the transformation of a mesogenous pathway to a perigenous pathway might accord with how information for stomatal ontogeny (conferred by the wall states, as well as by the regulation of λ and θ) is handed on during phylogeny. A switch from mesogenous to perigenous pathways could be perceived as a switch from the utilisation of information stored at the cell periphery to the utilisation of an inductive event which occurs as the result of the direct interaction between a meristemoid and a susceptible neighbouring P-cell. Remarkable in this respect is the unequal division in P-cells which neighbour meristemoids in leaves of *Zea mays* (Gallagher and Smith 1999; Cartwright et al. 2009) (see also Fig. 4). The meristemoidal cell body (Baluška et al. 2004) appears to induce the nucleus of a selected P-cell to secrete microtubule-organising material located on the external surface of its nuclear membrane (see also Baluška et al. 1997) and then physically deposit it at the cortical-cytoplasmic site where a pre-prophase band of microtubules will form and to which a new division wall will in due course attach (Gallagher and Smith 1999;

[11] It is remarkable that stomatal meristemoids have never been reported to produce directly a root or shoot apex; therefore, they do not seem to have the embryonic potentials suggested by Bünning (1956, 1965) (however, the property of "embryonality" – the term used by Bünning in the context of the meristemoid, appears to refer to the sites of synthesis of new cytoplasm in these cells, and even in the mother cell of the meristemoid). Leaf epidermal cells, and even guard cells, can be induced to divide (Tucker 1974), but they do so only within the confines of the leaf surface. Could it be that the tough, waxy surface of the leaf restricts meristemoid growth and division? But this is not to say that epidermal outgrowth can never occur: trichomes are one example where outgrowth does take place. Epiphylly is also well known in a few taxa. However, this last-mentioned process does not involve the stomatal meristemoids on the leaf surface but concerns mainly a sub-epidermal layer (see Brossard 1973). Yet, these sub-epidermal groups of cells do have "embryonality" since they are able to generate all the tissues of the new plantlet. They would also be meristemoids in Bünning's sense.

Cartwright et al. 2009; see also Panteris et al. (2007) for an account of the sub-cellular cytology of the *Zea* P-cell induction). And here, as suggested by Stebbins (1965a), intercellular pressures may also play a role (Palevitz 1982) as these can be one additional means by which nuclear movements are brought about (Kennard and Cleary 1997; Qu and Sun 2007).

10 Final Remarks

Transformational L-systems, such as we have applied to the ontogeny of stomata, are analogues of (or metaphors for) biological epigenetic processes. Such processes have been proposed to lie embedded within the macromolecular and physical structure of the cells walls and the peripheral cytoplasm (Barlow et al. 2001). Genetic systems, for sure, also have a role in stomatal ontogeny (Nadeau 2009): genes determine the rates at which macromolecules are manufactured, epigenetic systems determine how and where the resulting sub-cellular elements are assembled as functional constructions.

From the myriad stomatal types, and from the corresponding myriad ontogenetic pathways, it is difficult to discern (at least at first glance) whether one type has some peculiar advantage over another type. Energy requirements for stomatogenesis at the appropriate time of a plant's life cycle and in the particular circumstances of its habitat may be one consideration; another may be the period allotted for stomatogenesis within the plastochronic timescale prescribed for stem and leaf development. Nor should it be overlooked that stomatal complexes develop in three-dimensions (four, if time is included): the sunken stomata and the "floating" stomata of ferns draw attention to this third dimension of development.

The diversity of stomatal complexes also draws attention to the dearth of information about their overall physiology and function. Much attention, in this respect, has been paid to the perigenous stomatal complexes of monocotyledons (e.g. in cereals and Commelinaceae). Here, the role of the subsidiary S cells has been extensively examined in relation to stomata opening and closure. But the functioning of stomatal complexes, *as a whole*, is still something of a mystery for dicot plants. Even in the favourite plant, *A. thaliana*, the status of cells neighbouring the guard-cells is unclear. Could it be that S cells have different functions depending on their cellular descents (perigenous or mesogenous)? And could it be that the guard-cells and S cells are not the only epidermal cell types to move solutes in and out of their vacuoles? The response of guard-cells to this movement is the opening and closure of the stomatal pores: but this becomes possible only because of the differential wall thickening of the guard-cells and physico-mechanical differences between guard-cells and neighbouring epidermal cells (Sharpe et al. 1987). The critical solute exchange may occur not only within an areole domain (perhaps containing numerous stomatal complexes) but also between that domain and the veinlets which border its internal surface.

Further questions arise in connection with the distribution of stomata over the surfaces of leaves – i.e. the spatial aspect of stomatal complex ontogenesis. Although not much attention has been paid to this problem in the present chapter, stomatal clusters, such as found in *Begonia* spp., point not only to their interesting ontogenies but also to the possible advantages of such a type of formation (an advantage for *Begonia*, perhaps, but a possible disadvantage if displayed in other species). In addition, there is still the unaddressed question of whether or not the rhythm of stomatal opening and closure within a cluster of stomatal complexes is related to that of other clusters on the same leaf surface? These questions are intriguing in the light of non-synchronous (patchy) functioning of stomata-containing areolar domains (see Mott and Buckley 1998; Beyschlag and Eckstein 2001).

The study of stomatal complexes in its more global aspect is similar to present-day studies of genetics and physiology within the plant kingdom: a few selected forms or species are investigated intensively in the hope of finding enough data in these particular instances by which to extrapolate generalities. But much might remain hidden by such an approach (Bolker 1995). However, given that plant research is in its infancy (if one considers a potentially limitless future for Mankind's pursuit of knowledge of the natural world), consideration of stomatal development and function should be sufficient to awaken curiosity as to the significance of their complex features in relation not only to the relevant biological processes but also to the benefits which their study may have for agriculture and, hence, for human existence, as well as for the existence of the living world as a whole.

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Endoreduplication and Growth of Fleshy Fruits

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Contents

Abstract The fruit is a specialized organ, which results from the development of the ovary after successful flower pollination and fertilization, and provides a suitable environment for seed maturation and seed dispersal mechanisms. Due to

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their importance in human nutrition and their economic inference, fleshy fruit species have been the subject of developmental studies, mostly devoted to ovary formation, fruit set, and fruit maturation. The growth phase of the fruit has been much less addressed, although the complex interplay between cell division and cell expansion during this period is a crucial determinant of the final size, weight and shape of fruits. This chapter aims at reviewing our current knowledge on fleshy fruit development and addresses the cellular and molecular mechanisms involved in their growth, with a special emphasis on the cell expansion associated process of endoreduplication, with tomato fruit as the model species for fleshy fruits.

1 Introduction

The fruit is a plant organ specific to Angiosperms, which typically contains the seeds. At the botanical level, most fruits develop from mature ovaries and, therefore, include carpel tissues in part or whole. Additionally, many species develop mature fruit tissues from extracarpellary floral components, e.g., strawberry, pineapple, mulberry, and pome fruits (apple, pear), in which the receptacle, bracts, calyx, and floral tube (the fused base of floral organs), respectively constitute the majority of mature fruit tissue (Coombe 1976; Gillaspy et al. 1993; Nitsch 1953). The fruit has evolved in Angiosperms to fulfill ovule and seed protection during embryo development, and seed dispersal after maturation. This important physiological function accounts for a significant part of the adaptive success of Angiosperms. As such, the fruit has been under strong selective pressure, which accounts for the very wide diversity of fruit size, form and composition, and of seed and fruit dispersion mechanisms. These mechanisms range from the small, nondehiscent akene dry fruit, dispersed by wind, to the large, fleshy, and juicy berry and drupe fruits, which have to be eaten by animals, such as mammals or birds for seed dispersion and germination. As an example, the Solanaceae family, which encompasses nearly 10,000 species, has very diverse types of fruits, with capsules, drupes, pyrenes, berries, and several sorts of dehiscent noncapsular fruits occurring in more than 90 genera (Knapp 2002).

According to common use, fruit refers to the fleshy, edible fruits, such as grape, banana, tomato, citrus, cucurbits, pomes, stone fruit, and mango. These species are subjected to major agricultural production, which rely on permanent selection and improvement in yield and quality. Their common feature is tissues that accumulate water and many organic compounds, such as sugars, organic acids, pigments, flavor and aromas, and vitamins, which bestow their juiciness and attractiveness. Most fundamental knowledge exists about the control of maturation of fleshy fruit (Giovannoni 2001, 2004), and their postharvest handling (Brecht et al. 2003; Soliva-Fortuny and Martin-Belloso 2003), and more recently, about the processes involved in carpel morphogenesis and fruit set by using the model dry fruit of

Arabidopsis thaliana (Ferrandiz 2002; Ferrandiz et al. 1999; Roeder and Yanofsky 2006). Surprisingly, in the past 50 years, only a few reviews have addressed the molecular, cellular, and physiological events that control growth and differentiation in fleshy fruits (Bollard 1970; Coombe 1976; Gillaspy et al. 1993; Nitsch 1953, 1965, 1970; Varga and Bruinsma 1986). However, this developmental phase represents by far most of the total duration of fruit development, and is obviously associated with many parameters determining fruit quality, such as fruit size, shape, and composition.

Most recent studies on fleshy fruit growth have focused on tomato, Solanum lycopersicum (Gillaspy et al. 1993; Giovannoni 2004; Srivastava and Handa 2005; Tanksley 2004), a crop of the Solanaceae, which produces a multicarpellar berry. This crop is of strong economical importance, and for which numerous genetic (http://tgrc.ucdavis.edu/) and molecular (http://compbio.dfci.harvard.edu/tgi/) resources have been developed. In tomato, high levels of endopolyploidy occur in the course of fruit growth (Bergervoet et al. 1996; Bertin et al. 2007; Cheniclet et al. 2005; Joubès et al. 1999). This review deals with the cellular and molecular mechanisms involved in the growth of fleshy fruit, with a special emphasis on endopolyploidy and tomato fruit development.

2 Fruit Development and Growth

2.1 Carpel Morphogenesis and Fruit Set

Fruits typically develop from pre-existing organs, such as carpels inside flowers. The first phase of fruit development represents the morphogenesis and growth of carpels and ovules, from flower initiation to the double fecundation occurring in ovules (Gillaspy et al. 1993; Tanksley 2004). The ontogenic relationship between carpel and leaf has been emphasized (Gillaspy et al. 1993) and the genetic network responsible for carpel and ovule development has been thoroughly analyzed in *A. thaliana* (Ferrandiz et al. 1999). In grape, tomato, and apple, carpels are formed by ca. 17–20 rounds of cell divisions during this prebloom period, with virtually no cell expansion (Coombe 1976; Ho 1992). These divisions occur in the L3 layer of the floral meristem in a coordinated developmental pattern.

The number of cells formed in the ovary before anthesis is an important determinant of the potential final size in many fruits. This explains why fruits from early opening flowers are larger at maturity than those from later blooms (Coombe 1976). In addition, in many species including tomato (Bohner and Bangerth 1988a, 1988b; Frary et al. 2000; Tanksley 2004) and kiwi fruit (Cruz-Castillo et al. 2002), ovary size at anthesis and mature fruit size are frequently correlated positively. More work is needed to unravel the effect of internal cues,

e.g., sink effects (Ho 1992), and external ones, e.g., temperature (Bertin 2005; Higashi et al. 1999) on this phenomenon.

2.2 *Fruit Growth*

Shortly before anthesis, growth usually stops in the ovary. The second phase of fruit development, i.e., fruit growth, resumes only after pollination by compatible pollen and then fertilization (i.e., at fruit set). A major issue for understanding fruit growth is to decipher the signals and their mode of action, which simultaneously induce corolla and stamen senescence and fruit set after pollination and fertilization. Recent results in tomato suggest a function of abscisic acid (ABA) and ethylene before fruit set, to keep the ovary in a temporally protected and dormant state (Balbi and Lomax 2003; Vriezen et al. 2008).

The involvement of plant hormones in fruit set has long been postulated from the efficiency of externally applied auxins or gibberellins to replace pollination and fertilization in inducing fruit set and growth (Crane 1964; Nitsch 1965; Nitsch 1970; Vivian-Smith and Koltunow 1999). When the auxin signaling pathway is altered, fruit set and development can occur without fertilization, i.e., parthenocarpy (Carmi et al. 2003; Goetz et al. 2006; Goetz et al. 2007; Pandolfini et al. 2007; Wang et al. 2005). Auxin action on fruit set may also be through gibberellic acid (GA) synthesis (Serrani et al. 2008) and GA action (Martí et al. 2007). Fruit set has also been related to the strong increase of sucrose import capacity in young tomato fruit (Ho 1996; D'Aoust et al. 1999).

Fruit growth is by far the longest phase of fruit development. It ranges from 1 week for *A. thaliana*, 3–5 weeks for strawberry, 5–8 weeks for tomato, to 60 weeks for many citrus fruits with an average of 15 weeks for most fleshy fruits (Coombe 1976) (Table 1). Two distinctive types of fruit growth curves have been reported on cumulative and/or rate bases according to fruit species (Coombe 1976). Single S-shaped (sigmoidal) growth curves occur in most species including tomato. In few-seeded-fruit, such as stone fruit (drupes) and some berries, such as grape, growth curves fit a double-sigmoid pattern, which involves two successive phases of growth with physiologically distinct sink activity and a transition in between (DeJong and Goudriaan 1989). The type of growth curve for several fruit species is indicated in Table 1.

In general, fruit growth starts by a period of intense cell divisions. Then, before the frequency of cell division declines, cells begin to enlarge rapidly; the final period of fruit growth relies uniquely on cell expansion. As already pointed out more than 50 years ago, "fruit growth curves do not indicate when the transition of cell multiplication to cell enlargement occurs, so that, from the point of view of the growth process, distinction between both is not considered to be important. The important entity seems to be the organ rather than its constituents" (Nitsch 1953). Thus, although they offer convenient data for phenological studies, most growth curves are inadequate for the analysis of the components of growth (Coombe 1976).

Table 1 Characteristics of fruit growth in 22 species undergoing or not endopolyploidization, and sorted according to fruit growth duration

Family	Speciesenglish name	Speciesbotanical name	DNA contentpg/ 1C[a]	Dev. Curve[b]	Growth duration[c]	Max nb endo-cycles[d]	Cell diameter (m)[e]	References[f]
Brassicaceae	Arabidopsis	*Arabidopsis thaliana*	0.16	S	1	3	25	Vivian-Smith and Koltunow (1999)
Rosaceae	Strawberry	*Fragaria sp.*	0.4*	S	3–5	5	50	Suutarinen et al. (1998)
Cucurbitaceae	Melon	*Cucumis melo*	1.05	S	5–6	6	450	Kano (2007)
Solanaceae	Potato	*Solanum tuberosum*	0.88 or 2.10	n.d.	6	4	n.d.	-
Fabaceae	Common Bean	*Phaseolus vulgaris*	1.43	n.d.	6	4–5	n.d.	-
Cucurbitaceae	Cucumber	*Cucumis sativus*	1.77	S	6	6	>200	Boonkorkaew et al. (2008)
Solanaceae	Tomato	*Solanum lycopersicum*	1.0*	S	5–8	7–8	600–1,000	Cheniclet et al. (2005)
Solanaceae	Pepper	*Capsicum annuum*	4.00	S	8	6	800	Rygol and Lüttge (1983)
Rosaceae	Cherry	*Prunus avium*	0.35	D	9–11	4–5	65	Stern et al. (2007)
Rosaceae	Raspberry	*Rubus sp.*	0.3*	D	12	5	n.d.	-
Vitacaee	Grape	*Vitis vinifera*	0.43	D	14	0	350	Schlosser et al. (2008)
Rosaceae	Apricot	*Prunus armeniaca*	0.30	D	16	5	n.d.	-
Rosaceae	Peach	*Prunus persica*	0.28	D	16–26	5	120	Ognjanov et al. (1995)
Musaceae	Banana	*Musa sp.*	0.6*	Other	17–18	0	n.d.	
Rosaceae	Plum	*Prunus domestica*	0.93	D	19–22	4	100	Stern et al. (2007)
Rosaceae	Pear	*Pyrus communis*	0.55	S	18–25	0	n.d.	-
Rosaceae	Apple	*Malus domesticaor M. communis*	4.50	S	21–22	0	n.d.	-
Ebenaceae	Persimmon	*Diospyros kaki*	1.2–1.7*	D	21–28	0	220	Hamada et al. (2008)
Actinidiaceae	Kiwi	*Actinidia chinensis*	4.19	D	23–26	0	200	Hopping (1976)Cruz-Castillo et al. (2002)
Moraceae	Fig tree	*Ficus carica*	0.70	D	25	0	n.d.	-
Lauraceae	Avocado	*Persea americana*	0.93	S	40	0	n.d.	-
Rutaceae	Orange	*Citrus x sinensis*	0.63	S	60	0	n.d.	-

[a]DNA content per haploid genome (major source: Plant DNA C-values database, Royal Botanical Gardens, Kew, http://data.kew.org).
*mean of different varieties or data
[b]Type of development growth curve (S = single sigmoid, D = double-sigmoid)
[c]Length of fruit development from anthesis to ripe stage (in weeks) (literature and our personal data)
[d]Personal data on maximum number of endocycles undergone during fruit development. n.d.: non determined
[e]Approximate cell diameter of the largest mesocarp cells in mature fruit
[f]Main source of cell diameter data (p.d.: our personal data)

Fruit growth relies on a spatially and temporally organized pattern of cell division and of cell expansion. Remarkably, fleshy fruit tissues may have very various ontogenic origins (Coombe 1976), although they all share similar characteristics, with large, highly vacuolated cells with thin walls. In tomato, the ovary wall has seemingly a simple organization at anthesis, with ca. 11 layers of small isodiametric cells including two epidermal cell layers, and vascular bundles in the central pericarp (Cheniclet et al. 2005). During fruit organogenesis and growth, the fertilized ovary acquires a complex pattern of cells with various sizes and shapes, and various metabolic differentiations (Cheniclet et al. 2005; Gillaspy et al. 1993; Mohr and Stein 1969; Smith 1935) (Fig. 1a). How this spatio-temporal pattern of development is related to gene expression, metabolic profiles and cellular characteristics, such as endopolyploidy has only just started to be described (Lemaire-Chamley et al. 2005). This apparent slow progress has been due to the difficulty in correlating various cellular and molecular data at the level of given cell types in three-dimensionally complex organs, such as fruits. How this complexity relates to hormonal and nutritional regulation during fruit growth also remains largely ignored. Many data have been reported on the kinetics of hormone content in various fruit materials (reviewed in Gillaspy et al. 1993; Srivastava and Handa 2005), but their use for a proper understanding of their action in relation to growth mechanisms remains rather elusive. Fruit growth requires the combined presence of several growth-promoting plant hormones, such as auxin, gibberellins, cytokinins and brassinosteroids (Cowan et al. 2005; Ozga et al. 2002; Srivastava and Handa 2005). Many of these hormones appear to originate from the developing seeds, with a particular role for the endosperm in the secretion of these compounds.

2.3 Cell Division During Fruit Growth

Active cell division within the flesh is usually restricted to an initial period of 1–2 weeks after pollination and fertilization (e.g., cucurbits, tomato), 3 weeks in apple, 4–7 weeks in Japanese pear, peach, and plum. Cell divisions do not occur in the pericarp of Corynth grape, Rubus, or some Ribes species. However, divisions continue in avocado and in strawberry throughout the life of the fruit (Coombe 1976; Crane 1964; Nitsch 1965). In tomato and as in many fleshy fruits, cell divisions occur in various cells and with various division planes to allow fruit growth, but they also occur in discrete cell layers with definite division planes for specific purposes. This is the case for tomato, where the two epidermal cell layers of the pericarp undergo anticlinal divisions, whereas the two subepidermal layers undergo several rounds of periclinal divisions leading to an increase in the number of pericarp cell layers to a varying extent according to the tomato line. These various types of cell divisions are differently regulated because cell-layer forming cell divisions occur only within 5–8 days post-anthesis in various tomato lines (Cheniclet et al. 2005; Cong et al. 2002; Mazzucato et al. 1998), whereas

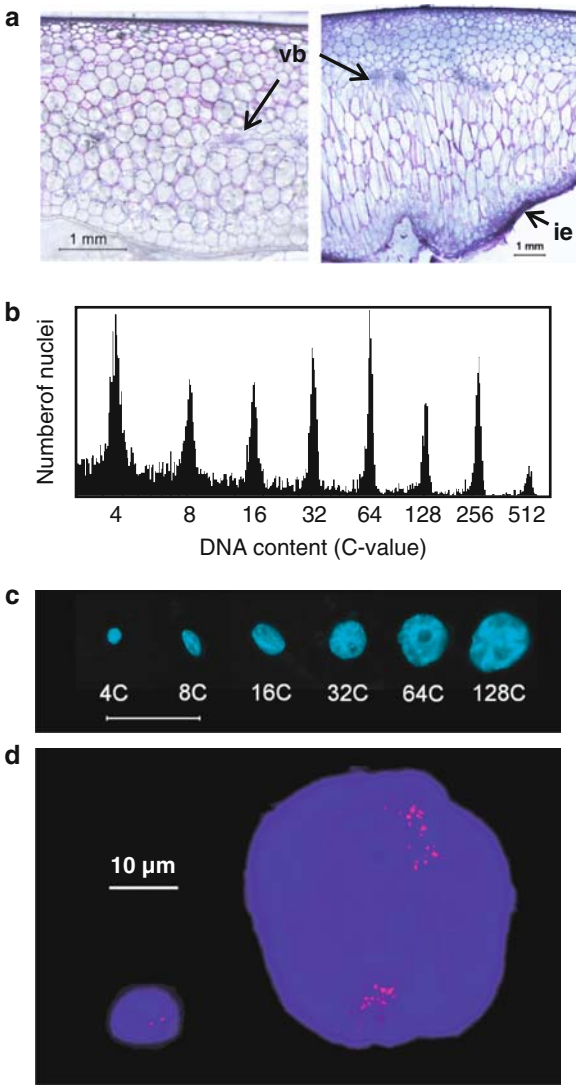

Fig. 1 Cellular aspects of endoreduplication in tomato fruit. (**a**) Pericarp histology in mature green fruits from two distinct tomato lines (*left*: Gardener's delight; *right*: Montfavet), showing the variability in pericarp tissue patterning resulting from differences in cell expansion. vb: vascular bundles; ie: inner epidermis. (**b**) Flow cytometry analysis of mature green fruit pericarp from a large-fruited line, showing the distribution of nuclei according to DNA content (C-value). (**c**) DAPI-stained nuclei isolated from mature green tomato pericarp (cherry line) and sorted according to their fluorescence intensity ; *from left to right*: 4C, 8C, 16C, 32C, 64C, 128C) ; note the increase in size and the increasing complexity of condensed chromatin distribution revealed by DAPI fluorescence. (**d**) FISH on two nuclei isolated from mature green pericarp tomato and flow cytometry sorted according to their ploidy class (*left*: 2C, *right*: 64C); DAPI-stained DNA appear in *blue*, and hybridization spots of a BAC probe specific for chromosome 7 appear in *red* after Texas-red revelation

randomly-oriented cell divisions occur for longer periods up to 10–18 days post-anthesis (Gillaspy et al. 1993; Tanksley 2004). Moreover, two different modified genetic backgrounds affect cell divisions in tomato pericarp, excluding cell-layer forming divisions (Cong et al. 2002; Jones et al. 2002). Commonly, tissues closest to the ovules (e.g., placenta in tomato or fruit surface in strawberry) cease division earlier than other tissues (Coombe 1976). All these data indicate spatially and temporally complex regulation of cell divisions in growing fruit.

After anthesis, the locular cavities in fruit are usually filled as a result of intense cell division activity from one or more of the locule surface areas (e.g., placenta in tomato, septum in banana and grape, endodermis in banana and orange, and seed aril in lychee). Cell expansion then contributes to the filling of the locule, which behaves in concert with the neighboring flesh to form fruit pulp or, as in tomato, to form a jelly-like tissue with distinct properties from the pericarp (Coombe 1976).

In conclusion, the fruit as a whole is composed of cells, which were present at anthesis and of newly formed cells during fruit growth. The ratio between both kinds of cells is a function of the number of doublings at these two phases. After anthesis, 80–97% of fruit cells in apple, strawberry, peach, apricot and tomato are produced, whereas ca. 70% of fruit cells are formed before anthesis in cucumber and blueberry (Cano-Medrano and Darnell 1997). The modulation of cell division in fruit either pre- or post-anthesis has repeatedly been associated with strong variations in fruit size. As an illustration, strong differences in overall anticlinal, but not periclinal, cell division in the pericarp are associated with varying levels of *fw2.2* transcripts corresponding to the major quantitative trait locus (QTL) for tomato fruit size, which accounts for as much as a 30% difference in fruit fresh weight between small-fruited and large-fruited tomatoes (Cong et al. 2002; Liu et al. 2003).

2.4 Cell Expansion During Fruit Growth

In eukaryotic cells, cell enlargement results from two processes: cell growth by increase in cytoplasmic volume and cell expansion through vacuolation. Cell growth by cytoplasmic volume increase may occur in all types of cells and is responsible for moderate increases in cell volume, by less than tenfold (Sugimoto-Shirasu and Roberts 2003). Cell expansion through vacuolation is a specific property of plant cells because of their large vacuolar compartment, and it leads to an increase in cell volume by more than one hundredfold (Sugimoto-Shirasu and Roberts 2003). Cell expansion through vacuolation typically starts in young organs, once cells have stop dividing, exit the cell cycle, and differentiate.

Some of the largest cells found in plants occur in the flesh of ripe fruit, with cell length between 150 and 700 μm, and in some cases more than 1 mm. The volume of cells in ovary wall is only ca. 10^{-3}–10^{-2} nL at anthesis whereas it is in the range of 1–10 nL, up to 100 nL in mature fruit (Coombe 1976). Because cell volume may be increased by 10^{2}–10^{4} times during the growth of fleshy fruit, this phenomenon

makes by far the greatest contribution to the total expansion of the fruit. Cell size has been recognized as a critical component of fruit size in cucumber, blueberry, and grape (Cano-Medrano and Darnell 1997).

When expressed on an arithmetic scale, cell expansion, by several orders of magnitude, has often been considered to occur after the cell division phase in fleshy fruit (Gillaspy et al. 1993). In fact, cell expansion starts in the very few days after fruit set, concomitantly with cell division (Boonkorkaew et al. 2008; Cheniclet et al. 2005), and it lasts for the entire period of fruit growth. However, whether initial cell growth occurs through cytoplasmic growth while the cells are actively cycling in the mitotic cycle remains poorly understood. The most intense cell growth phase then occurs through dramatic increases in the vacuolar volume and vacuolation index of fruit cells.

Cell size is typically described by linear cell dimensions, e.g., diameter or perimeter in fruit sections, which gives only an approximation of cell volume, and thus, requires deeper sectional analyses to take cell shape into account. The absolute measurement of cell size and shape in the growing grape fruit revealed that cell shape was irregular and cell volumes in the inner mesocarp of a grape berry exhibited a 14-fold range variation, with polysigmoidal distribution and clusterings around specific cell size classes (Gray et al. 1999). Obviously, more measurements of this type are required to fully understand the patterning of fleshy parenchymatous fruit tissues.

In the course of fleshy fruit development, the extent of cell expansion is influenced by cell wall behavior, turgor, and constraints imposed upon the flesh by the extensibility of the skin. Auxin is generally considered responsible for cell expansion during fruit growth, although this effect may not be direct but mediated through ill-defined compounds mediating sink activity.

3 Endopolyploidization

3.1 Definitions

Polyploidy can be defined as the addition of a complete set of chromosomes to one genome, which results from either sexual reproduction via 2n gametes or somatic chromosome doubling. According to the mode of polyploidy formation, allopolyploidy and autopolyploidy can be distinguished as originating respectively from interspecific hybridization between divergent progenitor species, thus giving rise to the presence of distinct subgenomes, and from intraspecific hybridization (or self-fertilization) or somatic chromosome doubling, thus resulting in identical subgenomes (Otto 2007). Related to autopolyploidy, endopolyploidy corresponds to the occurrence of different ploidy levels within an organism.

Endopolyploidy can result from the generation of multinucleate cells originating from acytokinetic mitosis, from nuclear fusion, from endomitosis, or from endoreduplication. Multinucleate cells in plants are found during the formation of the

syncitial female gametophyte (Yadegari and Drews 2004), in anther tapetum (D'Amato 1984) and seed endosperm (Berger 2003), while nuclear fusion may also contribute to polyploidization of the chalazal domain of the endosperm in Arabidopsis (Baroux et al. 2004). Endomitosis corresponds to a doubling of the chromosome number in nucleus. Chromosomes double and condense, sister chromatids separate normally, but return to the interphase state within an intact nuclear envelope, thus generating nuclear endopolyploidy. Although endomitosis mainly occurrs in animals, it is only rarely encountered in plants (D'Amato 1984).

Endoreduplication represents the most common mode of cell endopolyploidization in plants and is estimated to occur in over 90% of Angiosperms (Nagl 1976; D'Amato 1984). This process is an endonuclear chromosome duplication, which occurs in the absence of any obvious condensation and decondensation steps leading to the production of chromosomes with 2n chromatids without any change in chromosome number (Joubès and Chevalier 2000; Edgar and Orr-Weaver 2001). As a consequence, hypertrophying nuclei arise from successive cycles of DNA replication without segregation of sister chromatids, and in extreme cases "giant" polytene chromosomes are generated as observed for embryo suspensor cells of *Phaseolus* species (D'Amato 1984) or giant hair cells of *Bryonia* anthers (Barlow 1975).

3.2 Occurrence of Endopolyploidization in Fruit Species

As stated by D'Amato (1984), endopolyploidy is "of such a widespread occurrence in plants, that it can be regarded as the rule rather than the exception." A recent survey performed on several vegetative organs of 54 seed plant species different in term of genome size and belonging to two Gymnosperm and 14 Angiosperm families by Barow and Meister (2003) indicated that endopolyploidy occurred in 33 species taken from ten different families. Though absent in Gymnosperms, the frequent occurrence of endopolyploidy within Angiosperms seems restricted, however, to certain phylogenetic groups (Barow 2006).

Though largely documented in vegetative organs (Barow and Meister 2003), the occurrence of endopolyploidy in reproductive organs, especially in fruit tissues, and its extent in different species has gained little attention so far. Endopolyploidization was found in ovular tissues (antipodal cells, synergids, endosperm and embryo suspensor cells) and in anther cells (anther hair, glandular hair and anther tapetal cells), where endoreduplication seems to be the preferential way of polyploidization (D'Amato 1984; Carvalheira 2000; Bauer and Birchler 2006).

More than 60 years ago, endopolyploidization was reported initially in cucumber and other fruit materials (cited in Coombe 1976 and Barow 2006) and then in the mesocarp parenchyma cells in apricot (Bradley and Crane 1955). However, in this latter report the natural and/or physiological occurrence of the phenomenon in this fruit could be questioned as these authors observed polysomaty (increase in chromosome number according to endomitosis), after an auxin treatment and subsequent

needle wounding of the fruit mesocarp. More recently, some reports described the occurrence of endopolyploidization in the pericarp of *Sorghum bicolor* (Kladnik et al. 2006), *Ornithogalum umbellatum* ovary epidermal cells (Kwiatkowska et al. 2007), and sugar-beet pericarp (up to 32C in diploid and to 16C in triploid and tetraploid plants) (Lukaszewska and Sliwinska 2007). Most of the reports dealing with endopolyploidy in fruit so far concerned tomato (Bergervoet et al. 1996; Bertin et al. 2007; Cheniclet et al. 2005; Joubès et al. 1999) (Fig. 1b). On the contrary, endopolyploidy has been reported neither for grape (Ojeda et al. 1999) nor for apple (Harada et al. 2005).

To provide more data about the occurrence of endopolyploidization in fruits, we initiated a large scale analysis of ploidy levels in fruit of different species, focusing mainly on fleshy fruits. DNA content of nuclei isolated from whole ovaries at anthesis and from pericarp of fully developed fruits were analyzed by flow cytometry, and the maximum number of endocycles (corresponding to the number of DNA duplications, cf. Sect. 5.2) made during fruit development was determined. These data are presented in Table 1, together with additional parameters, such as the DNA content per haploid genome, the average cell diameter in fruit flesh (available data from the literature), and the type and duration of growth. The ordering of phylogenetic families and species within the table is based on the duration of growth accordingly.

From this table and additional data not shown, endopolyploidization in fruit appears to be dependent in part from the phylogenetic position of species, as observed for vegetative organs by Barow and Meister (2003). In some families (e.g., Rutaceae), no endopolyploidization was observed, while in others, such as in Brassicaceae, Cucurbitaceae, Fabaceae, and Solanaceae, most of the fruit species displayed a high degree of endopolyploidization. According to Barow and Meister (2003), a high frequency of endopolyploidization was also reported in vegetative organs of these families. Amongst Rosaceae, pome fruits (apple, pear) do not exhibit endopolyploidization, while stone fruits (*Prunus* sp.) as well as strawberry and raspberry undergo several rounds of DNA duplication.

Of the vegetative organs reported to display endopolyploidization, the number of endocycles that cells undergo is predominantly, 2–3, less frequently 4 and rarely 5 (corresponding respectively to ploidy levels of 8C, 16C, 32C and 64C for diploid species, where C is the DNA content of the unreplicated haploid genome of a gamete). Higher ploidy levels appear to occur more frequently in fleshy fruits for which 4–5 endocycles can often be observed (Table 1). In maize endosperm, fertilization gives rise to 3C cells, which undergo up to 5 endocycles in the course of development, resulting in a maximum ploidy level of 96C (Leiva-Neto et al. 2004). In fruits of Cucurbitaceae and Solanaceae, mesocarp cells commonly undergo 6 endocycles, the highest ploidy levels for these cells being reached in tomato where 8 endocycles (up to 512C) can be observed. This high level of endopolyploidy in tomato and the numerous data reported on this process in this species (Bergervoet et al. 1996; Joubès et al. 1999; Cheniclet et al. 2005) makes it an outstanding model for studying endopolyploidization and its physiological role during fruit development.

3.3 Cellular Aspects of Endoreduplication

3.3.1 Cell Size

Endopolyploidization and cell expansion often occur simultaneously in developing organs, and high ploidy level have long been reported as associated with large cell size (Joubès and Chevalier 2000; Sugimoto-Shirasu and Roberts 2003). An ultimate demonstration of a direct correlation between ploidy level and size in single cells requires simultaneous in situ determination of DNA ploidy level and size in individual cells. This has been achieved only in a limited number of vegetative tissues, such as leaf epidermis (Melaragno et al. 1993), hypocotyl (Gendreau et al. 1997), and symbiotic root nodules (Cebolla et al. 1999), and of floral tissues (Kudo and Kimura 2002; Lee et al. 2004). In tomato, Bünger-Kibler and Bangerth (1982) showed that the ploidy level in fruit pericarp cells increases from 4 to 10 days after anthesis. At this stage, small cells localized around vascular bundles and hypodermis display nuclei with a 4C level, while in the large parenchyma cells, the ploidy level was between 16 and 32C, thus arguing for cell enlargement that is correlated with DNA content. Subsequently, such an observation was also reported by Bergervoet et al. (1996) and Joubès et al. (1999), while Cheniclet et al. (2005) established clearly a direct correlation between mean cell size and mean C-level during the development of tomato fruit pericarp.

3.3.2 Nuclear Size

Obviously as the nuclear DNA content undergoes exponential amplification through endoreduplication, the nuclear volume is expected to increase accordingly. In tomato fruit, an increase in nuclear size was observed in most pericarp cell-layers in the course of development (up to 6-fold in diameter) (Bergervoet et al. 1996; Joubès et al. 1999). Nuclei sorted by flow cytometry according to their DNA content show a strong positive correlation between nuclear size and ploidy level as far as the lower ploidy levels are concerned, but this correlation becomes weaker for higher ploidy levels, due to a larger variation of nuclear sizes (Bourdon et al. personal communication) (Fig. 1c). The heterogeneity in nuclear size of highly polyploid nuclei suggests that the nuclear volume depends not only on its DNA content, but also on other parameters, such as the amount and conformation of nuclear proteins, RNA molecules, and the degree of chromatin condensation. Therefore, one cannot deduce the precise ploidy level of a nucleus merely from its relative volume in a given tissue. It is noteworthy that a positive relationship between nuclear size and ploidy level has also been demonstrated in various endoreduplicating plant tissues, such as metaxylem in maize roots, *Aloë arborea* (Agavaceae) and *Zebrina pendula* (Commelinaceae) (List 1963), endosperm in *S. bicolor* (Kladnik et al. 2006), and in species encompassing a wide variation in endopolyploidy (Barow 2006).

3.3.3 Nuclear Morphology

In most studies dealing with histological aspects of endopolyploidy, the overall morphology of nuclei has been observed in whole tissue or epidermal layers, but quite often the fine structure of nuclei was not described.

In tomato fruit, observations were made on mature pericarp nuclei by confocal microscopy, bright field microscopy and transmission electron microscopy (Frangne et al. personal communication). A gradual increase in nuclear size is observed between the epidermal cell-layer and the central cell-layers of pericarp. Nuclei also present differences in term of shape. In the epidermal cell layer, the nuclei display a regular shape, whilst in the central cell layers they acquire a complex shape, with numerous deep grooves and digitations. When these nuclei are observed with conventional fluorescence microscopy after DAPI-staining, the grooves in their DNA are barely visible, but are suggested by the complex distribution of condensed chromatin (Fig. 1c). The gradient of nuclear size and shape observed in situ from the epidermis to the central layers of pericarp is similar to the gradient observed in ploidy-sorted nuclei, thus strongly suggesting that the most central nuclei are the most polyploid. Whether or not this particular morphology of nuclei observed in tomato fruit results from their high ploidy level has not yet been determined. Such a variation in the shape of the nucleus according to ploidy levels has also been reported in different cell types of *A. thaliana* (Barow 2006).

3.3.4 Chromatid Organization in Fruit Polyploid Nuclei

As pointed out in Table 1, endopolyploidization is a widespread phenomenon in various fruit species. However to our knowledge, not a single report focused so far on elucidating the chromosome organization in endopolyploid fruits' cells, except the study of Bradley and Crane (1955), who demonstrated the occurrence of endomitoses in the mesocarp of apricot. In an effort to identify chromosome organization in polyploidy tissues of tomato fruit, we performed a fluorescence in situ hybridization (FISH) analysis of tomato pericarp nuclei sorted by flow cytometry according to their DNA content. A biotin-labeled bacterial artificial chromosome (BAC) DNA sequence specific for tomato chromosome 7 (O. Coriton, personal communication) was used as a probe.

As illustrated in Fig. 1d, a 2C nucleus displayed two spots of hybridization as expected for the two copies of chromosome 7, while the hybridization on a 64C nucleus resulted in a uneven distribution of spots throughout the whole nuclear volume, but a clustering in two separated areas. The spatial proximity of the hybridization spots within these two areas suggests that the sister chromatids stay closely associated during endopolyploidization, thus accounting for a polytene structure of chromosomes and consequently for endoreduplication in tomato fruit. Such polytenic structures were also observed in maize endosperm (Bauer and Birchler 2006), Arabidopsis root tips and mature leaves (Lermontova et al. 2006), and cabbage root tips (Sesek et al. 2005).

Even if polyteny seems to be widespread in various plant organs, differences in chromatin organization can be observed between cell types during endoreduplication. These differences lie mainly in a more or less complete pairing of sister chromatids and in the state of chromatin condensation. Sister chromatid pairing can be observed along the entire length of chromosomes, such as for giant hair cells in *Bryona* anthers (Barlow 1975) or just in part of them, such as the embryo suspensor cells in *Phaseolus coccineus* (Nenno et al. 1994). Recently, Schubert et al. (2006) provided evidence for a differential alignment of sister chromatids pairing along Arabidopsis chromosome 1, and thus, for polyteny in Arabidopsis vegetative tissues. In addition, the condensation state of centromeres in Arabidopsis endoreduplicated cells was shown to be variable and cell type-dependent (Fang and Spector 2005). Decondensation could also account for a greater range of chromatin movement in endoreduplicated cells (Kato and Lam 2003). Hence, further investigations are needed to determine the biological significance of chromosome and chromatin organization in endoreduplicated cells.

Since endoreduplication appears to be the preferred mode of endopolyploidization in most plant organs and notably in tomato fruit, we shall use this term from here onwards.

4 Proposed Physiological Roles for Endoreduplication

4.1 Endoreduplication and the Determination of Cell- and Organ Size

The frequent positive correlation between endoreduplication and cell size in many different plant species, organs, and cell types (Joubès and Chevalier 2000; Sugimoto-Shirasu and Roberts 2003; Kondorosi and Kondorosi 2004) is commonly interpreted as endoreduplication as the driver for cell expansion. The successive rounds of DNA synthesis during endoreduplication induce a consequent hypertrophy of the nucleus. This can influence the final size of the cell, which, therefore, may adjust its cytoplasmic volume with respect to the DNA content of the nucleus (according to the "karyoplasmic ratio" theory; Sugimoto-Shirasu and Roberts 2003). Cell expansion proceeds further through vacuolation, to adjust the size of the vacuolar compartment in accordance with the cytoplasmic volume, and even to increase the vacuolation index of the plant cell.

Cheniclet et al. (2005) provided evidence for such a positive correlation between cell size and ploidy level in pericarp tissue during fruit development. However insufficient comparison has been performed so far in other fruit species, and even in tomato, opposing results were reported in two tomato isogenic lines differing by fruit weight and sugar content QTLs (Bertin et al. 2003) and between large fruits of

lines cultivated at different temperatures (Bertin 2005). To gain further insight about the possible relationship between endoreduplication and cell size in fruit, we collected data from the literature and performed additional measurements of cell size in fully-grown fruit displaying or not displaying endopolyploidization (Table 1). This analysis indicated no clear-cut relationship between these two parameters. For example, nonendopolyploidizing fruits like kiwi, persimmon, and grape display cell diameters of ca. 200 μm, 220 μm, and 350 μm respectively, while smaller cell diameters are observed in endopolyploidizing fruits, such as the Arabidopsis silique (25 μm), strawberry (50 μm), cherry (65 μm), and peach (120 μm). Therefore the ability to develop large cells as measured by their diameters, is not restricted to endopolyploidizing fruits. Nevertheless when endopolyploidizing fruits are compared, it also appears that the largest cells are present in fruits that undergo the highest number of endocycles (diameters of 600–1,000 μm in tomato, 800 μm in pepper, and 450 μm in melon), which suggests that endopolyploidization might be necessary for plant cells to reach very large sizes.

However, various examples show that endoreduplication can occur in the absence of dramatic cell expansion. For instance, Gendreau et al. (1998) reported that a small cell size in some Arabidopsis mutants did not prevent the occurrence of the same number of endocycles as in larger cells present in wild-type. Endoreduplication is, thus, obviously not the only parameter either signifying or favoring cell expansion.

The mean level of endoreduplication of various plant organs has repeatedly been found correlated with organ size, as exemplified in Arabidopsis leaves (Cookson et al. 2006), pea cotyledons (Lemontey et al. 2000) and tomato fruit (Cheniclet et al. 2005). However, the function of endoreduplication in organ growth remains open to various hypotheses. Organ growth can be considered as the result either of a given balance of cell-based (autonomous) mechanisms relying on division, expansion, and endoreduplication or according to the opposite, of organismal level of regulation (Mizukami 2001; John and Qi 2008). In view of the organismal control, the synthesis of cytoplasm is the primary process and DNA endoreduplication would come second to maintain the karyoplasmic ratio (Cookson et al. 2006; John and Qi 2008). This latter parameter is difficult to estimate because of technical constraints, although it is a common observation that fruits with endopolyploidy such as tomato have larger protein contents linked to a larger cytoplasmic compartment, than fruits without endopolyploidy, such as apple (Harada et al. 2005).

Although the correlation between cell size and endoreduplication is obvious, which process triggers the other is still a matter of debate, as a kind of Chicken or the egg causality dilemma. Since endoreduplication corresponds to successive rounds of DNA duplication in the absence of mitosis as defined below (cf. Sect. 5), it is therefore likely that a minimal cell size must be required to commit to the following round of DNA replication, thus implying cell growth. However, once DNA synthesis is completed, the doubling of the DNA quantity can in turn promote cell growth, according to the "karyoplasmic ratio" theory (Sugimoto-Shirasu and Roberts 2003).

4.2 Endoreduplication and Cell Differentiation

In the model plant Arabidopsis, the influence of endoreduplication in forming large specialized cells was best characterized in epidermal cells of mature leaves (Melaragno et al. 1993), during hypocotyl development in which the ploidy levels vary according to light conditions (Gendreau et al. 1997), and in leaf single-celled trichomes (Larkin et al. 2007). The growth of trichomes was shown to be dependent on the succession of endocycles. The formation of a two-branched trichome cell requires three rounds of endocycle, leading to a 16C DNA ploidy level. A supplementary endocycle may eventually occur to give rise to the formation of a third branch and 32C DNA content. Moreover, cell growth and differentiation of trichomes is a genetically regulated process, since mutants affecting the nuclear ploidy level impacts positively or negatively on trichome cell size.

As illustrated for trichomes, endoreduplication often occurs during the differentiation of cells that are highly specialized in their morphology or metabolism. This is the case for cells from tomato fruit pericarp and jelly-like locular tissues (Cheniclet et al. 2005; Lemaire-Chamley et al. 2005; Chevalier 2007) as described above, for symbiotic host cells during the formation of nitrogen-fixing root nodules in legumes (Cebolla et al. 1999) and/or for the endosperm cells of maize kernels (Kowles and Phillips 1985; Kowles et al. 1990).

4.3 Endoreduplication and Metabolism

There are instances where endoreduplication is linked to endogenous metabolism. For example, nodule development on legume roots is initiated in response to interaction with the symbiotic bacterium *Sinorhizobium meliloti*. During their differentiation process, symbiotic nodule cells, programmed to fix nitrogen, develop into very large and highly endoreduplicated cells (Cebolla et al. 1999; Vinardell et al. 2003) and display an important transcriptional activity that is remarkably specific to the nodule (Mergaert et al. 2003). Also, in *Zea mays*, endosperm cells accumulate large amounts of starch and storage proteins, concomitantly with multiple and successive endocycles during seed development (Lopes and Larkins 1993).

Since a correlation exists between endoreduplication and cell differentiation-specific metabolism, it is tempting to speculate that one role of endoreduplication would be to modulate transcriptional activity by increasing the availability of DNA templates for gene expression as the gene copy number is obviously multiplied, and therefore, to modulate subsequent translational and metabolic activities. However, this hypothesis has neither been convincingly demonstrated nor negated in plant cells. For example, Leiva-Neto et al. (2004) showed that endoreduplication levels did not clearly impact on the expression level of some endosperm-specific genes, which led them to propose that endoreduplication in maize endosperm functions primarily to provide a store of nitrogen and nucleotides during embryogenesis and/or germination.

4.4 Endoreduplication in Response to Environmental Factors

An important physiological role of endoreduplication might be in the adaptation to adverse environmental factors, especially maintenance of growth under stress conditions. For instance, amplification of DNA may provide a means to protect the genome from DNA damaging conditions, such as uneven chromosome segregation or UV damage. In Arabidopsis, the *UV-B-insensitive 4* mutation (*uvi4*) promotes the progression of endoreduplication in hypocotyl and leaf development, and confers an increased UV-B tolerance (Hase et al. 2006). Endoreduplication was also demonstrated to be an adaptation factor in plant responses to high salt concentration (Ceccarelli et al. 2006). Moreover, an increase in the extent of endoreduplication reduced the impact of water deficit on epidermal cell size, leaf expansion rate and final leaf size (Cookson et al. 2006), and facilitated growth at low temperatures (Barow 2006), thus suggesting that an increase in DNA content can be of advantage in a given environment.

4.5 Endoreduplication and Growth Rate

As described above (cf. Sect. 4.1) endoreduplication does not seem to be necessary for cell expansion. However, it could participate in modulating the rate or organ growth and/or cell expansion. We previously demonstrated a clear correlation between mean ploidy level in fully developed pericarp and final fruit size and weight, when 20 tomato lines displaying a large range in fruit weight were compared (Cheniclet et al. 2005).

To investigate whether endoreduplication influences the fruit growth rate, we recorded the time of anthesis (full bloom in the case of trees) and fruit maturity (harvest time) for different fruit species and compared the determined length of development with the extent of endoreduplication (Table 1). From this analysis, it has been concluded that (1) in all species where fruit development lasts for a very long period of time (over 17 weeks), endoreduplication does not occur in fruit tissues; (2) in species displaying middle-length fruit development (10–16 weeks), some fruits do not display endoreduplication (e.g., grape), while others undergo 4–5 endocycles (e.g., fruits of many Prunus sp.); (3) in fruits, which develop rapidly (in less than 10 weeks) undergo several rounds of endocycle (3–8). Moreover, the formation of cells of similar size, and even larger ones, takes far less time in endopolyploidizing fruits than in nonendopolyploidizing fruits. Assuming that the ovary cells display roughly similar sizes at anthesis in the different species, these observations support the assertion that endoreduplication is likely to influence not only the rate of fruit development but also the rate of cell expansion in species exhibiting rapid fruit development. This would be consistent with the analysis of Barow (2006), who suggested that endoreduplication contributes to a greater extensive growth than in nonendopolyploid plants.

The rate of organ growth could be also under the influence of genome size. From the data presented in Table 1, no obvious relationship between genome size and length of fruit growth could be observed. For instance, pear and apple (nonendoreduplicating species) have respectively a small (C-value = 0.55 pg) and large (C-value = 4.50 pg) genome size and display similar fruit growth lengths of around 21 weeks. The same observation is also true for endoreduplicating species, such as tomato (C-value = 1 pg) and pepper (C-value = 4 pg), whose fruit both develops in 6–8 weeks. Thus, the positive correlation between genome size and life cycle shown by Bennett (1972) may not be applicable if confined to fruit development, at least in this range of genome size.

We also examined if the ability to exhibit endoreduplication was favored by a small genome size, owing to the fact that a higher nuclear DNA content as a result of autopolyploidy requires more time to replicate (Bennett 1972). From the 22 different fruit species analyzed in Table 1, it seems that the occurrence and extent of endoreduplication is not related to genome size, since endopolyploidizing fruits display genome sizes ranging from 0.16 to 4 pg and nonendopolyploidizing ones from 0.43 to 4.50 pg.This result is in accordance with the findings of Barow and Meister (2003) who observed only a low negative correlation between genome size and endopolyploidization, which was contrary to a previous claim based on fewer species (Nagl 1976).

5 Molecular Control of Endoreduplication

5.1 The Canonical Cell Cycle

The canonical eukaryotic cell cycle is composed of four distinct phases: an undifferentiated DNA presynthetic phase with a 2C nuclear DNA content, termed the G1 phase; the S-phase, during which DNA is synthesized, with a nuclear DNA content intermediate between 2C and 4C; a second undifferentiated phase (DNA postsynthetic phase) with a 4C nuclear DNA content, termed the G2 phase; and the ultimate M-phase or mitosis. Mitotic cell division is the ultimate step in the cell cycle, and leads to the transmission of the genetic information from one mother cell to two daughter cells. The classical cell cycle thus involves the accurate duplication of the chromosomal DNA stock in S-phase and its subsequent equal segregation in each nascent cell as a result of mitosis.

The progression within the four phases of the plant cell cycle is regulated by a class of conserved heterodimeric protein complexes consisting of a catalytic subunit referred to as cyclin-dependent kinase (CDK) and a regulatory cyclin (CYC) subunit whose association determines the activity of the complex, its stability, its localization and substrate specificity (Inzé and De Veylder 2006). The canonical A-type CDK (CDKA; Joubès et al. 2000) harboring the PSTAIRE hallmark in the cyclin-binding domain displays a pivotal role during the cell cycle as it participates

in different CDKA/CYC complexes to trigger the specific phosphorylation of numerous protein substrates at the boundaries between the G1 and the S phases, and between the G2 and M phases, thus allowing the commitment to DNA replication and mitosis respectively. The availability and binding of the regulatory cyclin subunit to a CDK partner is thus of prime importance for the regulation of the cell cycle. However, other levels of complexity in the regulation occur at the posttranslational level affecting the components of the CDK/CYC complexes. The kinase activity of the complexes is dependent on the phosphorylation/dephosphorylation status of the kinase itself, and also on the binding of CDK inhibitors and/or recruitment of additional regulatory factors (Inzé and De Veylder 2006).

5.2 The Endocycle

The endoreduplication cycle (endocycle) is made of the succession of S- and undifferentiated G-phase without mitosis, thus accounting for the cessation of cell division and the increase in ploidy level (Joubès and Chevalier 2000; Edgar and Orr-Weaver 2001; Vlieghe et al. 2007). As a result, part of the molecular control existing for the classical cell cycle regulation is conserved in the endocycle.

5.2.1 The Mitosis-to-Endoreduplication Transition

The commitment to endocycle and the consequent lack of mitosis has been proposed to occur in the absence of a mitosis inducing factor (MIF) which normally governs the passage through the G2-M transition. In addition, down-regulation of M-phase-associated CDK activity is sufficient to drive cells into the endoreduplication cycle (Vlieghe et al. 2007). The M-phase CDKB1;1 activity is required to prevent a premature entry in the endocycle. Hence, CDKB1;1 is the likely candidate kinase to be part of the MIF. Though the cyclin partner of CDKB1;1 inside MIF still awaits a definite identification, convincing in planta functional analyses highlighted the A-type cyclin CYCA2;3 to be the most appropriate candidate (Yu et al. 2003; Imai et al. 2006).

5.2.2 Relicensing of Origins of Replication

The control of MIF activity cannot account solely for the progression within the endocycle, since it requires the fluctuation in the activity of S-phase CDK between DNA synthesis and the undifferentiated G-phase as to allow relicensing of origins of replication. In eukaryotes, the initiation of DNA replication occurs only once during each cell cycle from multiple sites throughout each chromosome (Kelly and Brown 2000). These origins of replication trigger the assembly of prereplication complex (pre-RC) harboring several components, such as the ORC, CDC6, CDT1, and MCMs proteins (Bryant and Francis 2008). During the cell cycle, the control of the G1-to-S

transition in plant cells is exerted through the retinoblastoma-related protein (RBR) pathway (de Jager and Murray 1999; Gutierrez et al. 2002), where the hypophosphorylated form of RBR binds to the E2F-DP dimeric transcription factor, thereby repressing the E2F-responsive genes required for the commitment to the S-phase, such as those encoding the pre-RC components. Interestingly, the ectopic expressions of either *CDC6* (Castellano et al. 2001) or *CDT1* (Castellano et al. 2004), or E2Fa with its dimerization partner DPa (De Veylder et al. 2002) are sufficient to trigger extra endocycles, as well as the inhibition of RBR function, consistent with the inhibitory function of RBR on E2F (Park et al. 2005). It is noteworthy that the activation of DNA replication in all these transgenic plants results in extra endocycles and also causes prolonged cell proliferation activity. These data illustrate the conservation of molecular controls between the endocycle and the canonical cell cycle, but more importantly the importance of regulating the CDK activity, including the pivotal role of MIF at the onset of endoreduplication. It was thus proposed as a quantitative model that the amount of CDK activity controls the differentiation – and obviously the endoreduplication – status of a cell (De Veylder et al. 2007).

5.2.3 Regulation of CDK Activity in Endoreduplication During Fruit Development

Among the potential mechanisms regulating the CDK activities in endoreduplicating tissues, three distinct mechanisms are proposed to be affecting the components of the CDK/CYC complexes at the posttranslational level: (1) the WEE1 kinase regulates negatively the CDK activity by phosphorylation of residue Tyr15 before the commitment to mitosis as to ensure that DNA replication and repair on damaged DNA have been completed; (2) the active CDKA/CYCD complexes may be inhibited by specific CDK inhibitors, termed ICK/KRPs (for Interactor of cyclin-dependent kinase/Kip-related protein); (3) loss of CDK activity occurs upon the proteolytic destruction of the cyclin subunits via the ubiquitin proteasome pathway, involving the activation of the anaphase-promoting complex (APC) through its association with the CCS52.

Since endoreduplication plays such an important part during fruit development (Chevalier 2007), the relative contribution of these different control mechanisms on CDK activity has been addressed in tomato.

Role of WEE1

Homologues to *WEE1* have been isolated from various plant species (Shimotohno and Umeda 2007). While functional analyses performed in *Schizosaccharomyces pombe* indicated that expression of the maize or Arabidopsis gene led to the inhibition of cell division and significant cell enlargement (Sun et al. 1999; Sorrell et al. 2002), Arabidopsis knock-out mutants for *WEE1* grow perfectly well under nonstress conditions (De Schutter et al. 2007). Neither cell division nor endoreduplication was affected in these mutants, thus indicating that *WEE1* is not rate-limiting for cell cycle

progression under normal growth conditions. However, *WEE1* was shown to be a critical target of the DNA replication and DNA damage checkpoints, which operates in the G2 phase by arresting the cell cycle in response to DNA damage.

Since the *WEE1* gene is significantly expressed in endoreduplicating cells of maize endosperm (Sun et al. 1999) and tomato fruit tissues (Gonzalez et al. 2004), the function of the WEE1 kinase in the onset of endoreduplication *in planta* appears to conflict with the work of De Schutter et al. (2007). During tomato fruit development, i.e., in a highly endoreduplicating cell context, we provided evidence that *WEE1* is involved in the determination of endoreduplication and thus, participates in the control of cell size during tomato fruit development through its expected negative regulation on CDK activity (Gonzalez et al. 2007).

In Arabidopsis, the WEE1 Kinase controls the cell cycle arrest in response to activation of the DNA integrity checkpoint, thus targeting the CDKA/Cyclin complex, resulting in a stop of the cell cycle in the G2 phase until DNA is repaired or replication is completed (De Schutter et al. 2007). Unlike its Arabidopsis counterpart, the down-regulation of tomato *WEE1* induced a small-fruit phenotype originating from a reduction in cell size associated with a lowering of endoreduplication (Gonzalez et al. 2007), suggesting that *WEE1* acts on endoreduplication in a species-dependent manner, especially in the context of highly endoreduplicating tissues. Within the endocycles, a minimal cell size must be required to pass the checkpoint in the G-phase prior to the following round of DNA replication. Hence, WEE1 activity could contribute to inhibit the CDK/cyclin complexes driving the G-to-S transition, preventing a premature entry into the S-phase of the following endocycle by regulating the length of the G-phase and thus allowing cell enlargement (Gonzalez et al. 2007).

Role of ICK/KRP

Plant specific CDK inhibitors called ICK/KRPs bind and inhibit or sequester CDKs (Verkest et al. 2005b). In *A. thaliana*, when ICK/KRPs are constitutively expressed slightly above their endogenous level, only mitotic cell cycle specific CDKA;1 complexes are affected, thus blocking the G2/M transition, while the endoreduplication cycle specific CDKA;1 complexes are unaffected (Verkest et al. 2005a; Weinl et al. 2005). The fine tuning of the ICK/KRP protein abundance was demonstrated as a key feature for cell cycle control, and especially to trigger the onset of the endoreduplication cycle (Verkest et al. 2005a). The activity of the G2-to-M specific CDKB1;1 in dividing cells triggers the phosphorylation of Arath; KRP2, thus mediating its degradation by the proteasome, and consequently controls the level of CDKA;1 activity. Therefore, when CDKB1;1 activity decreases, Arath; KRP2 protein level increases, leading cells to enter the endoreduplication cycle.

Four different KRP inhibitors have been identified so far in tomato, namely Solly;KRP1 to Solly;KRP4. The respective expression of Solly;*KRP1* and Solly; *KRP2* displayed distinct behaviors, since Solly;*KRP1* is preferentially expressed at 20 DPA (both at the transcription and translation levels), when the endoreduplication process is maximal in the pericarp and the gel tissues, while Solly; *KRP2*

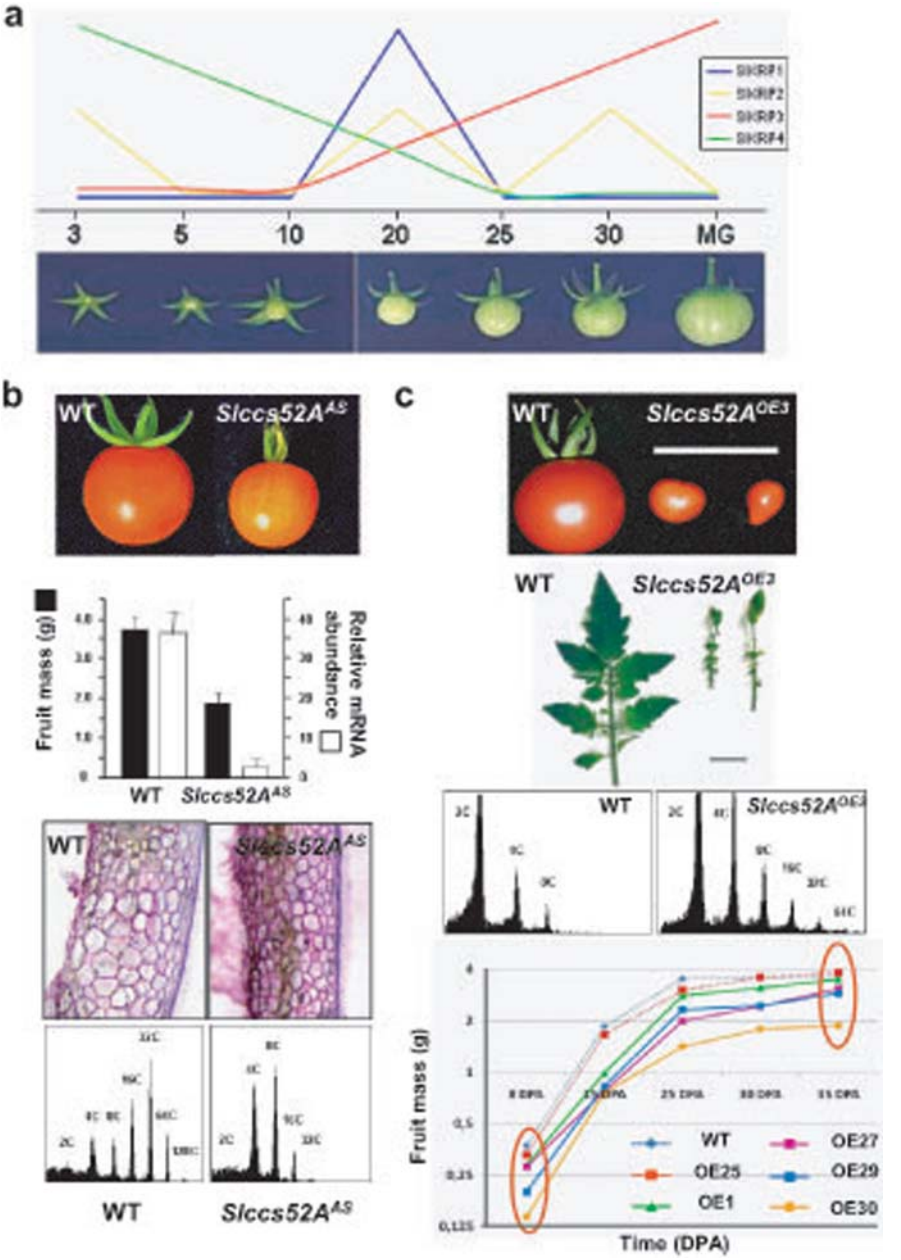

Fig. 2 Molecular and functional analysis of genes involved in the control of endoreduplication during tomato fruit development. (**a**) Schemed profile of mRNA expression for the four tomato KRP genes. The stages of fruit development are indicated in days post-anthesis. MG: Mature Green. (**b**) Phenotypic and molecular analysis of tomato *CCS52A* down-regulated *Pro35S: SlCCS52AAS* plants. Mature fruits (red ripe stage) were compared with untransformed control

expression peaks during fruit maturation (Bisbis et al. 2006). Using quantitative PCR following reverse transcription (RT-qPCR), we showed that the four tomato KRPs display unique expression profiles during tomato fruit development (Fig. 2a). Interestingly, Solly;*KRP4* is mostly expressed during the very early development of the fruit, i.e., when cell divisions predominantly drive fruit growth, while the expression of Solly;*KRP3* increases at the latest stages of fruit development, i.e., when cell expansion predominantly accounts for fruit growth. These data suggest that these KRPs may display distinct physiological and functional roles during the cell cycle and eventually, during the endoreduplication cycle.

During tomato fruit development, the large and hypervacuolarized cells constituting the jelly-like (gel) locular tissue undergo multiple rounds of endoreduplication. Within this particular tissue, mitosis is arrested after 20 DPA and only endoreduplication occurs until then concomitantly with a strong posttranslational inhibitory regulation of the CDKA activity (Joubès et al. 1999). The origin of this posttranslational regulatory mechanism resides in part in the accumulation of Solly; KRP1, which accounts for the inhibition of CDK/CYC kinase activity during the development of the gel tissue in tomato fruit (Bisbis et al. 2006).

Role of CCS52

The ubiquitin-dependent proteolysis of mitotic cyclins requires the involvement of a specific E3-type ubiquitin ligase named the anaphase-promoting complex/cyclosome (APC/C). In plants, the APC/C is activated by the CCS52A protein, homologous to the mammalian CDH1 and Drosophila FZR, which binds to cyclins to drive them towards the degradation process by the 26S proteasome (Capron et al. 2003). Like its eukaryotic counterparts, CCS52A was found to promote the onset and progression of endoreduplication (Cebolla et al. 1999; Vinardell et al. 2003). As far as the Arabidopsis CCS52A2 isoform is concerned, its transcriptional activity is under the control of the atypical E2Fe/DEL1 factor, which acts as a repressor of premature endocycle onset (Vlieghe et al. 2005; Lammens et al. 2008).

We have investigated the functional role of *CCS52* genes during tomato fruit development. Transgenic plants underexpressing *SlCCS52A* or *SlCCS52B* using the

Fig. 2 (continued) (WT) of the same age. Fruit mass measurements of 25 DPA-fruits harvested from *Slccs52A*^{AS} lines and WT. The corresponding transcript levels of 25 DPA-fruits were measured by real-time PCR, and the relative abundance of mRNA was normalized to Sl*βtubulin* and Sl*eiF4A*. Data are mean ± standard deviation ($n = 3$). The comparison of 25 DPA-pericarps from WT and *Slccs52A*^{AS} fruits reveals an alteration in cell size induced by the down-regulation of SlCCS52A, together with a shift towards lower DNA ploidy levels. (**c**) Phenotypic and molecular analysis of *Pro35S:SlCCS52AOE* plants overexpressing tomato *CCS52A*. Mature fruits (red ripe stage) and fully developed leaves from line *Pro35S:SlCCS52A*^{OE3} were compared with untransformed control (WT). In line *Pro35S:SlCCS52A*^{OE3} DNA ploidy levels in leaves were shifted towards greater levels as a result of CCS52A overexpression. The growth kinetics of fruits harvested from WT plants and *Pro35S:SlCCS52A*^{OE} plants (lines OE1, OE25, OE27, OE29 and OE30) was established by measuring fruit mass daily up to 35 DPA

CaMV 35S promoter as a primary approach were generated. While the Pro35S:*SlCCS52B*^{AS} plants showed no evident phenotype at all, the Pro35S:*SlCCS52A*^{AS} plants displayed smaller fruits than wild-type plants (Fig. 2b). The ploidy level of the Pro35S:*SlCCS52A*^{AS} fruits was reduced and correlated with a decrease in mean cell size and an increased cell number.

Gain-of-function transgenic tomato plants overexpressing *SlCCS52A* were also generated. In the most extreme case, the phenotype of the Pro35S:*SlCCS52A*^{OE} line OE3 exhibited gross changes in leaf development and morphology (Fig. 2c). The plant phenotype was characterized by the appearance of under-developed (small and curly) leaves, resembling those of Arabidopsis plants overexpressing CDK inhibitors (Wang et al. 2000; De Veylder et al. 2001; Schnittger et al. 2003), thus suggesting that cell division was deeply impaired in these plants. Interestingly, the ploidy level in these plants was increased towards high DNA levels. Five more *SlCCS52A* overexpressing plants were analyzed for which the overall phenotypes were less affected allowing subsequent analyses, especially fruit growth characteristics (Fig. 2c). In accordance with the level of *SlCCS52A* expression, which was not significantly different from that of WT, fruits from line OE25 did behave like WT. On the contrary, the growth of fruits from the four other lines expressing greater levels of *SlCCS52A* transcripts displayed a slower kinetics. However, fruits from these transgenic lines at 35 DPA tended to reach almost the same size as WT fruit.

To explain these data, the effects of a *SlCSS52A* overexpression during very early fruit development are essentially, likely to affect the cell division process thus leading to very small fruits. Thereafter, and in accordance with the functional specificity of *SlCSS52A* in the control of endoreduplication, fruit growth is then accelerated during the endoreduplication-driven cell expansion phase to recover an optimum final fruit size close to that of WT. A kinetic study of the appearance of highly polyploid nuclei during fruit growth in these Pro35S:*SlCCS52A*^{OE} lines does support strongly this hypothesis (Mathieu-Rivet et al. submitted).

The functional analysis of endoreduplication-promoting genes, such as *WEE1* and *CCS52A* thus demonstrates the physiological role of endoreduplication in fleshy fruit growth, since a reduction in cell size originating from a decrease in DNA ploidy levels impacts the whole fruit development and final fruit size. We cannot make short work of the above stated causality dilemma, and whether endoreduplication is as a driving regulator for cell expansion. Nonetheless *WEE1* is thought to control the endocycle G-phase length as to allow the sufficient cell growth prior to commitment to the next nuclear DNA amplification, and accordingly cell enlargement becomes determining for subsequent DNA reduplication. At the organ level, manipulating endoreduplication through *CCS52A* overexpression highlights the function of endoreduplication as an ultimate driving force for fruit growth, and even an enhancer of cell growth rate facilitating and/or accelerating fruit growth or bigger fruit size. As shown in our cytological analysis in Table 1, endoreduplication is strong when fruit development is short and, therefore, endoreduplication is a way to get big fruits rapidly as supported by our molecular data.

6 Concluding Remarks

Fleshy fruit organogenesis represents an interesting plant system to study at the molecular and functional level the regulation of cell division and cell expansion processes, and especially, the interplay between the classical cell cycle and the endoreduplication cycle. Among the different plant models, tomato is especially well suited to address this question. Indeed, the final size of the fruit depends upon both the cell number and cell size, which is strongly associated with endoreduplication. The originality of the tomato fruit model resides in the cellular and structural diversity of tissues that compose the fruit, and very importantly final cell size (cell diameter higher than 0.6 mm at the end of fruit development) and ploidy levels (up to 512C in some genotypes) (Cheniclet et al. 2005). Such levels of endopolyploidy represent values unmatched in other model plants, such as Arabidopsis or maize where the maximum physiological ploidy is limited respectively to 32C (epidermal cells of the hypocotyls) or 96C (seed endosperm). Why such high levels of endopolyploidy in tomato represents a crucial interrogation.

As reviewed in this chapter, several physiological traits have been attributed to endoreduplication in plants, and as far as fleshy fruits are concerned, the complete elucidation of the functional role of endoreduplication during their growth still awaits us. As discussed herein, we believe that endoreduplication facilitates the growth of fleshy fruits by driving an extended cell expansion process. Obviously, it is also tempting to speculate that endoreduplication is associated with cell differentiation and metabolic specialization cell in fruit cells in the course of fleshy fruit development. The biochemical composition of fruit is mainly determined during the growth period sustained by cell expansion: starch, organic acids, secondary metabolites and aroma precursors accumulate and thus contribute to the organoleptic and nutritional quality of fruit such as tomato. These metabolic modifications during fruit growth originate from a profound change in the cellular behavior and gene expression program of fruit cells and are concomitant with endoreduplication as recently analyzed (e.g., Lemaire-Chamley et al. 2005; Mounet et al. 2009). However, the direct link between endoreduplication and gene expression control still awaits a definitive demonstration.

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Physiology

Adaptation to a Changing Environment: The Regulatory Role of Small RNAs

Dortje Golldack, Ines Lüking, and Ulrike Süthoff

Contents

Abstract Plants respond to changes in their abiotic and biotic environment with the activation of a complex network of adaptive mechanisms that particularly include cellular signaling and stress-responsive gene expression. More recently, plant microRNAs (miRNAs) and small interfering RNAs (siRNAs) have been shown to function not only in plant development but also as important modulators of plant stress responses. Both stress-induced expressions of miRNAs, the presence of stress-relevant *cis*-elements in the promoters of miRNA genes as well as target prediction showed that miRNA-mediated post-transcriptional events are regulatory mechanisms in triggering the responses of plants to a variable environment. Many miRNAs control regulatory networks of plant environmental adaptation by targeting transcription factors and might integrate both cellular signaling and adaptive mechanisms via hormonal responses and biosynthesis of phytohormones.

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1 Introduction: Small RNAs are Regulators in the Adaptation of Plants to Various Stress Conditions

Adaptation of plants to their environmental conditions as the nutrient status and abiotic stresses such as low temperature, drought, and salinity involves the activation of complex intracellular signaling cascades, regulation of gene expression and diverse acclimatory biochemical and physiological changes. The induction of specific cellular signaling, the activation of different transcription factors, specific transcription and translation play a central role in the environmental adaptation. During the last years, the picture emerged, however, that also post-transcriptional events are regulatory mechanisms that have an essential function in triggering the responses of plants to variable environmental conditions. Thus, the transcription and translation of developmentally and environmentally responsive genes might be regulated on the post-transcriptional level for example by the group of RNA-binding proteins (RBP) as well as by different classes of small RNAs as microRNAs (miRNAs) and small interfering RNAs (siRNAs).

RNA-binding proteins are known for the regulation of mRNA stabilization and turnover as well as mRNA localization via binding to the non-translated regions of mRNAs and play thus an important role in plant development, signalling, and responses to biotic and abiotic stresses (Chinnusamy et al. 2008). As an example, the glycine-rich RNA-binding protein GRP7 that is one of the eight members of glycine-rich RNA-binding proteins (GRPs) in *Arabidopsis thaliana* is involved in the export of mRNAs from the nucleus to the cytoplasm under cold stress conditions and confers freezing tolerance mediated by the regulation of stomatal opening (Kim et al. 2008).

As it was recently discovered, miRNAs and siRNAs are another group of modulators of gene expression on the post-transcriptional level by gene silencing via specific repression of mRNA translation or by mediating mRNA degradation. miRNAs have an essential function in the control of plant development as the regulation of flower, meristem, and vascular development. Thus, for example the regulatory factors *CUP-SHAPED COTYLEDONS* (*CUC*) and the gene products of *REVOLUTA* (*REV*) and *PHAVOLUTA* (*PHV*) as well as *PHABULOSA* (*PHB*) that are involved in meristem development are regulated by miRNAs (Williams et al. 2005; Chen 2005). More recently, the regulatory function of miRNAs in plant adaptation to environmental stresses as cold, heat, salt and dehydration as well as the plant nutrient status, mechanical stresses, biotic attacks and phytohormone-mediated signalling is now emerging.

2 The miRNA Machinery

miRNAs have been first described as a regulatory element of larval development in the nematode *Caenorhabditis elegans* in 1993 and have been discovered since in a wide variety of metazoans and plants as well as bacteria and amoeba (Lee et al.

1993; Millar and Waterhouse 2005; Gottesman 2005; Hinas and Söderbom 2007). Mature miRNAs are 20–24 nucleotides in length with a predominant size of 21 nucleotides (for example Reinhart et al. 2002; Bartel 2004). The miRNAs are derived from longer single stranded primary miRNA transcripts (pri-miRNA) that form imperfect hairpin-like secondary structures (Fig. 1). The miRNAs are released from the pri-miRNAs by the RNase III-like endoribonulease Dicer-like 1 (DCL1), and they are methylated by the action of the HUA ENHANCER1 (HEN1) protein, which contains a methyltransferase domain and transfers a methyl group from S-adenosylmethionine to the miRNA duplexes (Tkaczuk et al. 2006). Single-stranded miRNAs are subsequently integrated into cytoplasmic RNA-induced silencing complexes (RISC). In addition to DCL1, the dsRNA-binding protein HYPONASTIC LEAVES1 (HYL1) and the C2H2 Zn-finger protein SERRATE (SE) are necessary for the accurate processing of miRNAs that particularly accelerate DCL1-mediated cleavage (Dong et al. 2008).

In the active RISC the miRNAs serve as a template for post-transcriptional silencing of complementary mRNAs by inhibition of translation or by degradation of the target mRNA. Next to miRNA, the cytosolic RISCs contain argonaute proteins that are characterized by the presence of PIWI and PAZ domains. The PIWI domain is similar to an RNase H domain whereas the PAZ domain enables the binding of small RNAs by direct interaction (Wang et al. 2008).

siRNAs are another group of small RNAs that are functionally involved in post-transcriptional gene regulation. In contrast to miRNAs, the 20–24 nucleotide long siRNAs are derived from double-stranded RNA by the ribonucleases DCL2, DCL3 and DCL4. The methylated single-stranded siRNA is integrated in a RISC or in RITS (RNA-induced transcriptional silencing complex) that contain also argonaute proteins and are involved in histone methylation and heterochromatin assembly (Iida et al. 2008). Three main subclasses of siRNAs are known: trans-acting siRNAs (ta-siRNA), repeat-associated siRNAs (rasiRNA), and siRNA derived from natural antisense transcripts (nat-siRNA). Interestingly, proteins in the CPL1 family encoding an RNA polymerase II C-terminal domain (CTD) phosphatase contain both a phosphatase catalytic domain and double-stranded RNA (dsRNA)-binding motifs (DRM) and have recently been shown to interact with transcription factors in abiotic stress signaling (Bang et al. 2008).

Although miRNAs are supposed to function as one of the most important regulatory molecules of posttranscriptional control of gene expression, there are also other cellular mechanisms of posttranscriptional regulation. Gene expression might be also controlled by, for example, chromatin modifications, splicing of mRNA, and polyadenylation of transcripts. Up to now, it can not be estimated to what degree the different mechanisms contribute to the posttranscriptional regulation of gene expression (Chen and Rajewsky 2008; Zhang et al. 2007). It is not known yet, what are, for example, the differences in their functional rates and their efficiency of action in plant cells. Accordingly, it will be an important goal for future analyses of the function of miRNAs to elucidate the specific roles of miRNAs and of other regulatory mechanisms in posttranscriptional controls within cellular regulatory networks.

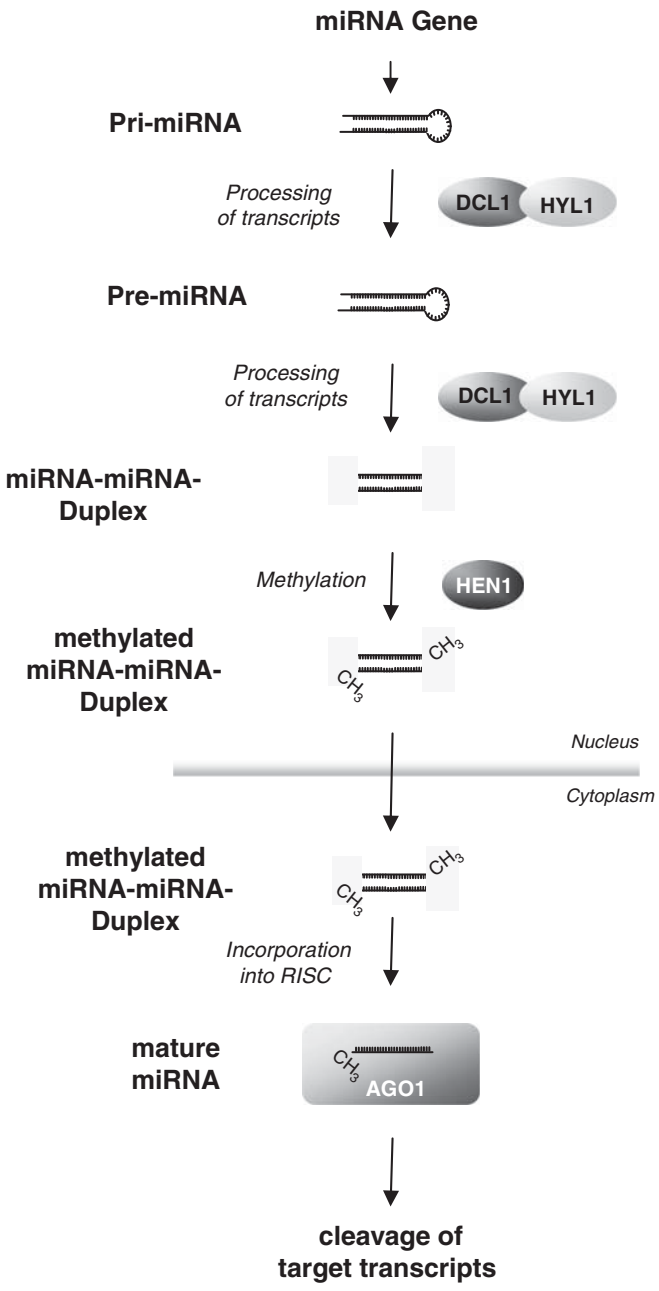

Fig. 1 Model for biogenesis and function of miRNAs in *Arabidopsis*

Several approaches have been established for the identification and the transcriptional analysis of miRNAs. Bioinformatical tools are used for the large scale prediction of miRNAs by computational analysis (Rabani et al. 2008). In addition, a number of experimental techniques have been adapted to specifically investigate small RNAs. These methods include Northern hybridizations, RT-PCR based amplification of miRNAs, cloning, and *in situ* hybridizations (Obernosterer et al. 2006; Lau et al. 2001; Chen et al. 2005; Gonzalez-Estevez et al. 2009). In addition, miRNA-specific oligo-microarrays and SAGE-based techniques (serial analysis of gene expression) can be applied for analysis of miRNAs (Yin et al. 2008; Cummins et al. 2006). For a bead-based profiling, miRNA-specific capture probes are attached to beads that are labeled with fluorescent dyes. cDNA that is generated from miRNAs can bind to the beads and in a flow cytometer the fluorescence intensity can be monitored for both detection and quantification of specific miRNAs (Lu et al. 2005; Kong et al. 2009). Another attractive method for miRNA detection and quantification is the RNA-primed array-based Klenow enzyme assay (RAKE). For RAKE, miRNAs bind to complementary specific oligonucleotides that are linked to a glass matrix. By exonuclease I, unbound single-stranded oligonucleotides are degraded and the bound miRNAs serve as a primer in subsequent PCRs. In the PCR, biotin-conjugated nucleotides serve as a label for signal amplification and allow identification of the bound miRNAs (Nelson et al. 2006).

3 The Functional Role of miRNAs in Plant Responses to Abiotic Stress

3.1 miRNAs and the Adaptation to Cold and Heat

Although the presence of conserved miRNAs has been predicted from a wide variety of plant species ranging from mosses and ferns to higher plants, most knowledge on plant miRNAs and their function has been derived from the model plants *A. thaliana* and rice (*Oryza sativa*) and the deciduous tree poplar (*Populus trichocarpa;* Lu et al. 2008b). Particularly, the developmental role of miRNAs as for example the involvement of miRNAs in the *NAC* transcription factors *CUC1*- and *CUC2*-mediated regulation of both vegetative and floral development in *A. thaliana* has been well studied (for example Chen 2005). Meanwhile, however, also the important function of miRNAs in the plant adaptation to abiotic and biotic stress conditions is emerging.

Changes in temperature are abiotic environmental factors with a significant impact on plant development and yield. Whereas low temperatures cause for example membrane rigidification, lamellar-to hexagonal II phase transitions of membranes, dehydration of cells and generation of active oxygen species, heat exposure leads mainly to solubilization of membranes that affects processes as the photosynthesis and photoinhibition (for example Thomashow 2001; Xiong et al. 2002). Next to

model plants as *A. thaliana*, the presence and functional role of temperature-induced miRNAs has been recently well studied in poplar (*P. trichocarpa*). In a large-scale approach, Lu et al. (2008b) generated a small-RNA library from *P. trichocarpa* plants that were exposed to different abiotic stresses and they identified 68 putative miRNA sequences that were classified into 27 miRNA families. Among these small RNAs, 19 cold stress-responsive miRNAs from 14 miRNA families were identified from which 15 were up-regulated and four were down-regulated in response to cold treatment. The predicted target genes of the cold-responsive miRNAs included homologues of the *A. thaliana* IAA receptors, transcription factors of the *NAC* and *GRAS*-type suggesting function of the miRNAs in regulation of signaling pathways and stress-dependent gene expression under control of specific *cis* acting elements (Lu et al. 2008b; compare also Table 1). Interestingly, expression of a number of miRNAs was not only changed by cold but also by heat exposure in a similar way indicating that the responses of plants to both low and elevated temperatures might be regulated by

Table 1 Examples of miRNAs that regulate stress-specific transcripts in plants

Stressors	miRNA	Species	Predicted targets	References
Cold	miR482	*P. trichocarpa*	Disease resistance protein	Lu et al. (2008b)
	miR1444	*P. trichocarpa*	Polyphenol oxidase	Lu et al. (2008b)
Heat	miR482	*P. trichocarpa*	Disease resistance protein	Lu et al. (2008b)
	miR1444	*P. trichocarpa*	Polyphenol oxidase	Lu et al. (2008b)
	miR1447	*P. trichocarpa*	Disease resistance protein Beta-fructofuranosidase Oxidoreductase	Lu et al. (2008b)
UV-B	miR172	*A. thaliana*	Glycosyl transferase	Zhou et al. (2007)
	miR398	*A. thaliana*	Cytochrome *c* oxidase	Zhou et al. (2007)
Salt	miR1447	*P. trichocarpa*	Disease resistance Beta-fructofuranosidase Oxidoreductase	Lu et al. (2008b)
Dehydration	miR1447	*P. trichocarpa*	Disease resistance protein Beta-fructofuranosidase Oxidoreductase	Lu et al. (2008b)
Mechanical stress	miR1444	*P. trichocarpa*	Polyphenol oxidase	Lu et al. (2008b)
	miR1445	*P. trichocarpa*	Dihydropyrimidinase	Lu et al. (2008b)
	miR1448	*P. trichocarpa*	Glutathione S-conjugate ABC transporter (MRP2) Disease resistance protein ATP-binding cassette tranport protein	Lu et al. (2008b)
Nutrition	miR395	*A. thaliana*	ATP sulphurylase (APS) Sulphate transporter (AtSULTR2;1)	Chiou (2007)
	miR398	*A. thaliana*	Copper/zing superoxide dismutase	Yamasaki et al. (2007)
	miR399	*A. thaliana*	Ubiquitin-conjugating E2 enzyme *PHO2*	Lin et al. (2008)
Pathogens	miR156	*P. taeda*	Disease resistance protein	Lu et al. (2008a)
	miR407	*P. taeda*	Beta-1,3-glucanase	Lu et al. (2008a)
	miR420	*P. taeda*	Peroxidase	Lu et al. (2008a)

convergent regulatory mechanisms. For example, down-regulations by cold and heat were observed for miR1450 that has a leucine-rich repeat transmembrane protein kinase as a target suggesting that adaptation to both low and high temperatures might be regulated by partly shared signaling pathways. In contrast, miR1447 showed reduced expression levels in response to heat and dehydration but was not significantly changed by cold. The targets of the miR1447 family in poplar include an ankyrin repeat family protein, a fructofuranosidase, and an oxidoreductase indicating a miRNA-mediated regulation of cellular signaling, metabolism, and defense (for example Becerra et al. 2004).

3.2 *miRNAs and the Response to Excess Light and Oxidative Stress*

Light is essential for plant life by supplying the energy source for photosynthesis. In addition, light that is sensed by photoreceptors of the classes of red/far-red light absorbing phytochromes, and the blue light absorbing cryptochromes and phototropins has a key function in regulating developmental processes (Frankhauser 2001; Briggs et al. 2001; Franklin et al. 2005). Excess light intensities have, however, stress effects on plants by straining the photosynthetic electron transport and the chloroplast metabolic reactions (Niyogi et al. 2005). Excess light is one of several abiotic stressors that are closely linked with the generation of reactive oxygen species (ROS). Under high light, excess electrons might be transferred to O_2 thus generating the superoxide anion radical ($O_2^{\bullet -}$). $O_2^{\bullet -}$ can subsequently react to hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and H_2O by acting as oxidant in single electron transfer reactions. ROS may damage for example membranes by lipid peroxidation and changes of transmembrane ion fluxes, by activation of oxidative bursts that lead to ROS-induced programmed cell death (Loreto et al. 2001; Loreto and Velikova 2001). In response to high light intensities plants activate protective mechanisms that include down-regulation of the transcript levels of genes encoding for the two photosystems, the light harvesting antenna, ferredoxin and ferredoxin-$NADP^+$ reductase as well as the cytochrome b_6f complex (Kimura et al. 2003). Reactive oxygen species might be detoxified intracellularly by enzymatic antioxidants as for example glutathione peroxidases and peroxiredoxins by disulfide formation of the compounds with a second thiol group (for example Alscher et al. 2002; Oelze et al. 2008).

Although the biochemical, physiological, and molecular responses of plants to excess light and reactive oxygen species have been studied in detail, only recently an involvement of posttranscriptional regulation of transcripts in the adaptation to these stressors became clear. In the moss *Physcomitrella patens*, for example, miRNAs were identified by analyzing the genomic sequences of cloned small RNAs for miRNA precursor-like structures, and putative target genes of the miRNAs were predicted (Talmor-Neiman et al. 2006). Among these targets, homologs of a phytochrome/light sensor protein kinase and a photoreceptor as well as a cytokinin

receptor and a NAC transcription factor were identified thus indicating that in the moss *P. patens* miRNAs have a functional role in both light and cytokinin signalling (Talmor-Neiman et al. 2006). In *A. thaliana*, UV-B responsive miRNAs were identified by a computational approach including the presence of known light-relevant *cis*-elements in the promoters of miRNA genes and upregulation of 21 miRNAs could be detected this way (Zhou et al. 2007). Interestingly, eight of the 11 putative light-inducible miRNAs were targeting transcription factors as auxin response factors, MYB factors, and a scarecrow-like transcription factor whereas a target gene of for example miR398 is a cytochrome c oxidase (Zhou et al. 2007). The results presented in this study support the findings that many miRNAs target transcription factors and thus are regulatory factors of a wide range of down-stream networks of gene expression (Bartel 2004; Zhou et al. 2007). Other targets of miR398 are the cytosolic Cu/Zn superoxide dismutase CSD1 and the chloroplastic CSD2 that are both involved in detoxification of superoxide radicals (Sunkar et al. 2006). miR398 was down-regulated in response to oxidative stresses, and *A. thaliana* over-expressing a miR398-resistant CSD2 was more tolerant to high light, heavy metals, and other oxidative stresses suggesting that stress-induced accumulation of the enzyme is post-transcriptionally regulated by miR398 (Sunkar et al. 2006).

3.3 The Role of miRNAs in the Plant Adaptation to Salt and to Dehydration

Exposure to excess salt results in accumulation of salt within the cytoplasmic compartment that causes ionic stress and damage of the plant metabolism. In addition, high apoplastic salt concentrations lead to osmotic stress and dehydration of the plant. Plants adapt to salinity by maintaining the intracellular ion homeostasis via regulated influx and efflux of Na^+ and Cl^- ions at the plasma membrane and by vacuolar ion sequestration (Hasegawa et al. 2000).

Salt-induced transport processes include the regulation of Na^+-influx by transcriptional control of HKT1-type Na^+-transporters and AKT1-type K^+-channels, maintenance of intracellular Cl^- homeostasis by CLC^--type Cl^- channels as well as vacuolar sequestration of Na^+ by the vacuolar H^+-ATPase and the tonoplast NHX1-type Na^+/H^+-antiporter (Apse et al. 1999; Golldack and Dietz 2001; Rus et al. 2001; Golldack et al. 2002, 2003; Diedhiou and Golldack 2006). Cytoplasmic synthesis and accumulation of osmoprotectants as polyols and sugars, proline, and quaternary ammonium compounds as well as regulation of water fluxes across plasma membrane and tonoplast enable cellular osmotic adjustment, prevent cellular dehydration and maintain structural stabilization of proteins and membranes (Popova et al. 2003; Kirch et al. 2000). Recently, important knowledge has been derived on signaling elements and transcription factors involved in the plant adaptation to salinity and dehydration. These include for instance the SnRK-type serine-threonine protein kinase SOS2 that was mainly characterized from *A. thaliana* and the Ca^{2+}-dependent protein kinase *OsCDPK7* that improved salt

tolerance in rice (Liu et al. 2000; Xiong et al. 2002; Saijo et al. 2000). Increased tolerance to drought was for example achieved by over-expression of the bZIP transcription factors *ABF3* and *ABF4* (*AREB2*) and the *NAC* transcription factors *ANAC019* and *ANAC055* whereas a function of the *C2H2*-type zinc finger proteins *STZ*, *AZF1*, *AZF2*, and *AZF3* as transcriptional regulators has been suggested in salt-stressed plants (Choi et al. 2000; Uno et al. 2000; Kang et al. 2002; Tran et al. 2004; Sakamoto et al. 2004). Whereas the transcriptional responses described as aforementioned have been well documented meanwhile from different research groups, an involvement of posttranscriptional processes inclusive a function of regulatory small RNAs has only been emerging recently. As one example, expressional analyses of miRNA417 in *A. thaliana* under stress conditions showed regulation of the small RNA by salt stress, dehydration stress, or abscisic acid (ABA) treatment (Jung and Kang 2007). Transgenic *A. thaliana* plants over-expressing miRNA417 showed retarded seed germination in the presence of high salt or ABA suggesting that miRNA417 acts as a repressor of seed germination under salt stress conditions (Jung and Kang 2007). Sunkar and Zhu (2004) exposed seedlings of *A. thaliana* to different abiotic stresses inclusive salinity and cloned miRNAs from small RNA libraries. Expressional analyses showed up-regulation of miR393 that has the F-box protein TIR1 as a putative target by cold, dehydration, NaCl, and ABA. In general, the F-box protein TIR1 is involved in auxin-mediated signalling and cellular responses (Salmon et al. 2008; Hayashi et al. 2008). In addition, also miR397b that has a casein kinase II as a putative target and miR402 putatively targeting a DNA glycosylase ROS1-homolog were slightly up-regulated by the stress treatments. In contrast, miR389a.1 with an unknown protein as a target showed down-regulation by the abiotic stresses tested (Sunkar and Zhu 2004).

Using microarray data, Liu et al. (2008) identified miRNAs that are regulated specifically by salinity, drought, and cold as well as miRNAs that responded to all three stressors. miRNAs that were regulated under all three stress conditions were miR168, miR171, and miR396 that have ARGONAUTE1 and SCL and GRL transcription factors as targets (Liu et al. 2008). Whereas miR408 with a peptide chain release factor as a target was specifically inducible by drought, several other miRNAs as for example miR159 where responsive to salt and have different transcription factors as MYB and TCP transcription factors as targets (Liu et al. 2008). These results demonstrate that different abiotic stressors as drought, salt and temperature changes activate an integrating cellular regulatory network of gene expression with miRNA-mediated targeting of partly same transcription factors that require a fine tuning by interactions of the convergent and divergent signaling ways.

3.4 miRNAs Function in the Adaptation to Mechanical Stress

In addition to identification and functional characterization of miRNAs in herbaceous plants as *A. thaliana* and the crop plant rice, interesting knowledge on miRNAs and post-translational regulation of gene functions has been derived

from the deciduous tree *P. trichocarpa*. Next to analyses of miRNA generation in response to the abiotic stresses cold, heat, salt and drought, plant responses to mechanical stresses have been one focus of the research during the last years. Mechanical stresses as wind and gravity require continuous developmental and metabolic adaptation of trees by the biosynthesis of cell wall components and by development of specialized cell and tissue types (Hu et al. 1999). These adaptational processes include the balancing of the biosynthetic pathways of cellulose, hemicellulose, and lignin that are the major components of woody tissue by either suppressing or stimulating the gene expression involved in the synthetic reactions (Hu et al. 1999; Lu et al. 2005; Lu et al. 2008b). In addition, mechanical stimuli lead to developmental adaptation that is mainly the regulation of cambial tissue, xylem, and fiber cell growth and development in wooden tissue (Lu et al. 2005). Lu et al. (2005) isolated miRNAs from developing secondary xylem of *P. trichocarpa* stems and identified both miRNAs that are conserved among plant species as well as miRNAs that are absent from herbaceous models as for example *A. thaliana*. Interestingly, even conserved miRNAs showed specific developmental expression in *P. trichocarpa* and *A. thaliana* that leads to the hypothesis that homologous miRNAs may have different regulatory functions in different plant species (Lu et al. 2005). The functional targets of the majority of identified miRNAs could be predicted by computational analyses and included mainly genes with functions in the biosynthesis of cell wall metabolites, development, and defense. Up-regulation in tension-stressed developing xylem could be for example shown for ptr-miR474 that has a protein kinase as predicted target. In contrast, down-regulation could be shown for example for ptr-miR478 and ptr-miR482 that have an organic anion transporter-like protein and a disease resistance protein as putative targets (Lu et al. 2005). Recently, Lu et al. (2008) identified additional stress-responsive miRNAs from *P. trichocarpa* that included small RNAs that are responsive to mechanical strains, and their mechanical stress-responsive expression was analyzed in developing xylem exposed to tension. ptc-miR1444 regulating a polyphenol oxidase belongs for example to up-regulated miRNAs whereas ptc-miR1450 targeting a leucine-rich repeat transmembrane protein kinase showed down-regulation in response to mechanical stress demonstrating also a functional role of miRNAs in regulation of signaling pathways in mechanically strained wooden tissue (Lu et al. 2008b).

4 miRNAs and Plant Nutrition

To ensure growth and development, plants have to acquire essential mineral nutrients via their root system from soil and have to balance distribution and transport of the elements among tissues and cells. Inorganic phosphate is one of the essential plant macronutrients and is often limited in supply for the reasons of low concentrations in the soil and of immobility due to covalent and non-covalent binding within the soil substrate (e.g. Marschner 1995). The understanding of

uptake and assimilation of phosphate was greatly advanced by identification and characterization of *A. thaliana* mutants that are impaired in uptake and allocation of the element. The *pho1* mutant shows defects in loading inorganic phosphate into the xylem that cause symptoms of phosphate deficiency in the shoots (Poirier et al. 1991; Hamburger et al. 2002). In contrast, *A. thaliana pho2* mutants show enhanced uptake and transport of the element leading to over-accumulation of phosphate in shoots (Delhaize and Randall 1995; Dong et al. 1998). The *PHO2* gene encodes an E2 ubiquitin conjugase (*UBC24*) that is expressed in roots and leaves of *A. thaliana* in vascular cells except the mature xylem (Bari et al. 2006; Aung et al. 2006). Interestingly, expression of *PHO2* and thus maintenance of phosphate homeostasis is controlled by miRNA399 as it was shown by *A. thaliana* over-expressing miR399 (Fujii et al. 2005; Bari et al. 2006). In a time course experiment, induction of miRNA399 was shown after 24 but not 12 h of phosphate starvation whereas its putative target *UBC* showed down-regulation starting at 24 h (Fuji et al. 2005). In another study, Liu et al. (2008) investigated the time-dependent modifications of transcript levels of ten different miRNAs in response to NaCl, mannitol, and low temperature. In response to salt and mannitol, the majority of analyzed miRNAs showed changed transcript amounts at 12–24 h after the onset of the stress whereas the cold treatment resulted in a changed transcription at 6 h of low temperature. These data demonstrate that posttranscriptional regulation of gene expression by miRNA is not a very fast cellular response but might rather have a role in long-term adaptation to changed environmental conditions.

As it was recently shown, miRNA399 is a phloem-mobile long distance signal that controls phosphate homeostasis by regulating *PHO2* transcript levels (Pant et al. 2008). Accumulation of miRNA399 was induced by phosphate starvation in the phloem sap of *Brassica napus* and of *Cucurbita maxima* and increased specifically in response to phosphate but not for example sulfate deprivation (Pant et al. 2008). Based on results of long-distance movement of miRNA399 in phloem but not xylem it was suggested that miR399 might systemically suppress *PHO2* by movement from shoots to roots and thus regulate uptake and translocation of phosphate (Lin et al. 2008).

Other examples of miRNA-mediated long distance regulation of mineral supply are the maintenance of copper and sulfate homeostasis. Presence and response to nutrient deprivation was shown for miR396 that is involved in sulfate supply and miR398 with a function in copper homeostasis in phloem but not xylem of *B. napus* (Buhtz et al. 2008). miR398 regulates copper homeostasis in *A. thaliana* by targeting a subunit of the mitochondrial cytochrome *c* oxidase and by controlling the degradation of copper/zinc superoxide dismutase transcripts under copper limiting conditions (Yamasaki et al. 2007). Interestingly, Dugas and Bartel (2008) found also an increased accumulation of miR398 and reduced mRNA and protein of copper superoxide dismutases *CSD1* and *CSD2* in *A. thaliana* grown on sucrose. These results suggest that not only copper but also sucrose availability may derive molecular signals that regulate *CSD1* and *CSD2* expression via miR398 (Dugas and Bartel 2008) that has for example also a functional role in oxidative stress tolerance as described above.

The other aforementioned nutrient, sulphur, has a central function in the cellular metabolism being a component of amino acids, enzymes, and by participating in redox processes with glutathione and thioredoxin (Chiou 2007). In *A. thaliana*, the uptake of sulphate is mediated by the high affinity transporters SULTR1;1 and SULTR1;2 as well as the low-affinity transporters SULTR2;1 and SULTR2;2 (Takahashi et al. 2000; Maruyama-Nakashita et al. 2003). Intracellularly, sulphate is metabolized and assimilated via for example the enzyme ATP sulphurylase (APS) (Chiou 2007). The miRNA395 functions in sulphate assimilation by controlling the transcript levels of the sulphate transporter AtSULTR2;1 and the ATP sulphurylase (Jones-Rhoades and Bartel 2004; Chiou 2007).

5 The Function of miRNAs in Response to Biotic Attacks

Next to a wide range of abiotic stressors, plants are also challenged by biotic pathogen attacks by microbes as for example bacteria, fungi, and viruses and react by local and systemic defense mechanisms (Hammond-Kosack and Jones 1996; Durrant and Dong, 2004). As a primary response plants recognize microbe-associated molecular patterns (MAMPs) by MAMP receptors, and both intracellular defense cascades as well as responses in the extracellular matrix are induced to limit the growth of pathogens (Bittel and Robatzek 2007). The non-host resistance enables plants to resist to a wide range of pathogens by restricting penetration via the cell wall and by intracellular defense mechanisms as for example the production of reactive oxygen species, of ethylene, and modification of ion fluxes that are activated by pathogen-responsive gene expression and involves MAP-kinase and jasmonate/ethylene signaling (Navarro et al. 2004; Zimmerli et al. 2004; Agorio and Vera 2007; Bittel and Robatzek 2007; Robatzek et al. 2007; Lipka et al. 2008). Gene products with a reported function in the non-host resistance of plants are for example *NHO1* encoding a glycerol kinase, PEN1 syntaxin and PEN2 glycosyl hydrolase (Kang et al. 2003; Lipka et al. 2005; Pajonk et al. 2008).

Although it seems likely that small RNAs have not only a central function in adaptation to abiotic stresses as reported above but might also regulate the responses of plants to invasion by pathogens, up to know, however, the knowledge on pathogen-induced miRNAs and their role in biotic stress resistance is quite fragmentary. Involvement of miRNAs in the response of *Pinus taeda* L. (loblolly pine) in response to infection with the endemic rust fungus *Cronartium quercuum* f. sp. *fusiforme* that causes the fusiform rust disease in pines has recently be shown by Lu et al. (2008). The authors of the study cloned and identified 26 miRNAs from the developing stem xylem of *P. taeda* and showed modified transcriptional expression of the majority of these miRNAs in galled stem tissue. Putative targets of the pine miRNAs include transcription factors and signaling elements as for example scarecrow-like and MYB transcription factors as well as a calcium-dependent and a LRR protein kinase (Lu et al. 2008a). On the other hand, transcripts encoding peroxidases, β-1,3-glucanase, and disease proteins have be predicted as targets suggesting

miRNA and target interactions both in signaling as well as in defense reactions within the process of rust gall development (Lu et al. 2008a).

In *A. thaliana* it was shown that the peptide flg22 derived from bacterial flagellin that acts as a pathogen-associated molecular pattern induces miR393 (Chisholm et al. 2006; Navarro et al. 2006). miR393 negatively regulated the transcripts for the F-box auxin receptors TIR1, AFB2, and AFB3, and interestingly antibacterial resistance was enhanced by repression of auxin signaling (Navarro et al. 2006). Suppression of the miRNA pathway in the miRNA deficient mutants *dcl1-9* and *hen1-1* sustained growth of non-pathogenic bacterial strains demonstrating that miRNAs have a central function in the non-host resistance of plants (Navarro et al. 2008). In addition, Navarro et al. (2008) identified bacterial effectors that suppressed miRNAs indicating that bacteria evolved mechanisms to suppress RNA silencing and may thus cause diseases. Mutation in ARGONAUTE4 enhanced host and non-host pathogen susceptibility in *A. thaliana* indicating that RNA-directed DNA methylation in the process of transcriptional gene silencing is required for resistance to pathogens (Agorio and Vera 2007). Recently, long siRNAs (lsiRNAs) that are 30–40 nucleotides in length have been identified as a novel class of bacteria-induced small RNAs (Katiyar-Agarwal et al. 2007). AtRAP1 that is a RAP-domain protein involved in disease resistance is a target of AtlsiRNA-1 and may be destabilized by mRNA decapping and 5′–3′ degradation (Katiyar-Agarwal et al. 2007).

6 miRNAs Control the Function of Transcription Factors and Phytohormones

Diverse stress-responsive miRNAs have been identified that target genes with specific functions in abiotic and biotic stress adaptation and defense. In addition, many miRNAs also target, however, transcription factors and components of signaling pathways and thus the miRNAs participate to a large extend in controlling regulatory networks and adaptive signaling cascades. Stress-adaptive gene expression in plants is mainly achieved by combination and interaction of various signal transduction pathways with regulation by specific and shared *cis*- and *trans*-acting factors. The transcription of many genes that are responsive to abiotic stress is controlled by ABA signalling pathways, and ABA-responsive elements (ABRE) as well as the dehydration-responsive element (DRE; C-repeat element (CRT)) have been identified in promoters of abiotic stress responsive genes (Shinozaki et al. 2003). DRE-binding proteins are ERF/AP2-type transcription factors as for example the cold-responsive CBF1 (DREB1B), CBF2 (DREB1C), and CBF3 (DREB1A), whereas DREB2 is induced by drought and salt stress (Shinozaki et al. 2003). In addition to dehydration and salinity, ABA-responsive *cis* elements (ABRE) respond to endogenously induced redox and metabolite signals and to the second messenger Ca^{2+} (Pastori et al. 2003; Rolland et al. 2006; Kim et al. 2004; Kaplan et al. 2006). Group A bZIP and AP2-related transcription factors can regulate ABRE

and ABRE-like elements and integrate exogenously induced signalling cascades with endogenously, hormonally triggered signalling pathways (for example: Choi et al. 2000; Uno et al. 2000; Kang et al. 2002).

Regulation of class II HD-ZIP AP2-type, MYB, and auxin response factors (ARF) by miRNAs has been particularly identified in the context of plant development. Class III HD-ZIP transcription factors direct for example the polarity establishment in leaves and vasculature and AP2-type factors have a role in flower development whereas MYB transcriptions factors are involved in leaf development (Bowman 2004; Chen 2004; Palatnik et al. 2003). The picture emerges, however, increasingly, that miRNA-mediated posttranscriptional regulation of transcription factors plays a central role in adaptation of plants to abiotic and biotic stress conditions (Table 2). In maize root cells submergence-responsive miRNAs were identified that have ARFs, HD-ZIP, WRKY, and scarecrow-like transcription factors as targets (Zhang et al. 2008). In response to fusiform rust disease, the defense-related miRNA targets in loblolly pine included MYB, AP2/ERF and scarecrow-like transcription factors (Lu et al. 2008).

In *A. thaliana* it has been shown that miR393 targeting the F-box protein TIR1 is up-regulated not only by cold, dehydration and NaCl but also by treatment with ABA (Sunkar and Zhu 2004). Expression of miRNA417 was modified by salt, dehydration, and ABA and it was suggested that miRNA417 acts as a negative regulator of seed germination (Jung and Kang 2007). Accumulation of miR159 is induced by ABA and regulates the transcript level of its targets *MYB33* and *MYB101* indicating that these transcription factors are regulators of ABA signaling in a miR159-dependent way (Reyes and Chua 2007). As another example for miRNA-controlled hormonal responses, expression of TCP transcription factors is modulated by miR319 and regulates jasmonate biosynthesis, leaf development and senescence (Schommer et al. 2008). Interestingly, the *A. thaliana hyl1-1* mutant showed impaired responses to auxin, cytokinin, and ABA with decreased sensitivity to auxin and cytokinin but hypersensitivity to ABA indicating that elucidation of far more interconnections of plant hormone signaling and regulation of and by miRNAs within the cellular network of environmental adaptation may be expected (Lu and Fedoroff 2000).

7 Summary and Outlook

Results of studies reviewed above demonstrate that same signalling pathways in response to abiotic and biotic stresses as well as acquirement of nutrients may be fine-tuned by different small RNAs on the post-transcriptional level. miRNAs modulate adaptive mechanisms to changes in the plant environment by regulating stability and life-time of transcription factors and phytohormones as well as the biosynthesis of hormones. In addition, also classes of stress-specific regulatory proteins as enzymes, transporters or defence proteins are regulated post-transcriptionally by miRNAs

Table 2 Examples of miRNAs regulating transcription factors and phytohormones under stress conditions

Stressors	miRNA	Species	Predicted targets	References
Cold	miR530	*Populus trichocarpa*	Zinc knuckle (CCHC-type) family protein Homeobox transcription factor KN3	Lu et al. (2008b)
	miR1450	*P. trichocarpa*	Leucine-rich repeat transmembrane protein kinase	Lu et al. (2008b)
Heat	miR530	*P. trichocarpa*	Zinc knuckle (CCHC-type) family protein Homeobox transcription factor KN3	Lu et al. (2008b)
	miR1447	*P. trichocarpa*	Leucine-rich repeat transmembrane protein kinase	Lu et al. (2008b)
	miR1450	*P. trichocarpa*	Leucine-rich repeat transmembrane protein kinase	Lu et al. (2008b)
UV-B	miR159	*A. thaliana*	MYB transcription factor	Zhou et al. (2007)
	miR160	*A. thaliana*	Auxin response factor	Zhou et al. (2007)
	mir170	*A. thaliana*	Scarecrow-like transcription factor	Zhou et al. (2007)
Salt	miR1447	*P. trichocarpa*	Leucine-rich repeat transmembrane protein kinase	Lu et al. (2008)
	miR171	*A. thaliana*	Scarecrow-like transcription factor	Liu et al. (2008)
Dehydration	miR1447	*P. trichocarpa*	Leucine-rich repeat transmembrane protein kinase	Lu et al. (2008)
	miR167	*A. thaliana*	Auxin response factors	Liu et al. (2008)
	miR171	*A. thaliana*	Scarecrow-like transcription factor	Liu et al. (2008)
Mechanical stress	miR159	*P. trichocarpa*	MYB transcription factor	Lu et al. (2005)
	miR169	*P. trichocarpa*	Auxin response factor	Lu et al. (2005)
	miR159	*P. trichocarpa*	Scarecrow-like transcription factor	Lu et al. (2005)
Pathogens	miR159	*P. taeda*	MYB transcription factor	Lu et al. (2008a)
	miR171	*P. taeda*	Scarecrow-like transcription factor	Lu et al. (2008a)
	miR172	*P. taeda*	APETALA2 transcription factor	Lu et al. (2008a)

(Fig. 2). Identification of conserved miRNAs and their targets in different plant species will help to understand conserved mechanisms of post-transcriptional gene silencing as a prerequisite for miRNA-based approaches of genetically engineering increased abiotic and biotic stress tolerance in plants.

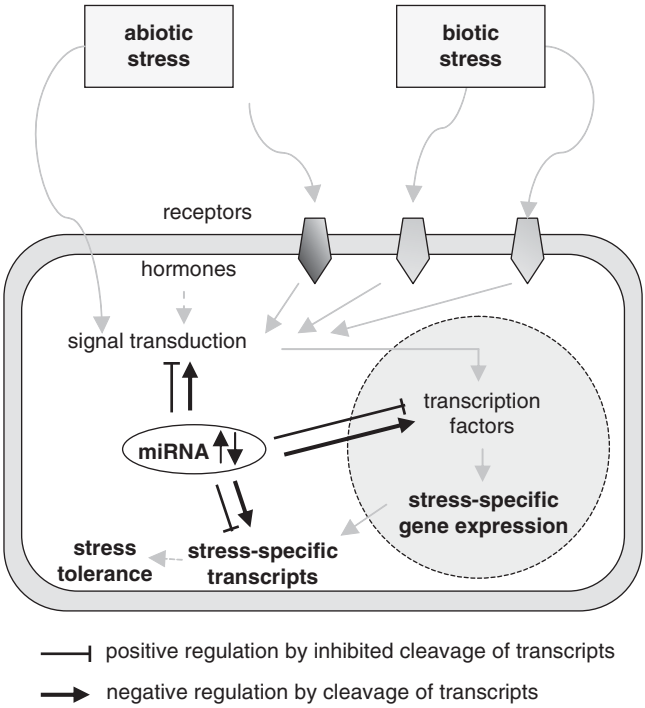

Fig. 2 Model of miRNA-mediated regulation of target genes in response to abiotic and biotic stresses in plant cells

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Growth Responses of Trees to Arctic Light Environment

K. Taulavuori, M. Sarala, and E. Taulavuori

Contents

Abstract Currently, climate is warming at a considerable rate. The warming climate shifts the vegetation toward the poles. This causes competition between southern and northern plant species and ecotypes. Light environment obviously has an important role in controlling the competition. The controlling factors include daylength, spectral composition (especially the ratio of red/far-red light, blue light and UV-radiation), and the amount of photosynthetically active radiation.

1 Climate Change and Light Environment

Temperature is a major factor controlling the geographical distribution of plants, affecting plants via both the temperatures of the growing season (e.g., Dahl 1951) and the cold winter period (Sakai and Larcher 1987). For example, the Arctic treeline coincides approximately with the +10°C isotherm of the mean temperature

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of the warmest month (e.g., Grace et al. 1989), and it determines the success in sexual reproduction. However, there is a wide heterogeneity of tolerance to low temperatures in the plant kingdom. Species from high latitudes are extremely winter hardy because of their extracellular freezing mechanism, while plants from the temperate zone are not as tolerant to below zero temperatures.

Currently, climate is changing at an exceptionally high rate (e.g., ACIA 2005; IPCC 2007). The warming climate shifts the "climatic envelopes" towards the poles. To survive in the projected climate change, species must either adapt to the new climate or move towards the poles or higher altitudes to keep themselves within their climatic envelope (Walther et al. 2002). Consequently, species from south shift their ranges northward, in some cases by as much as 1,000 km (ACIA 2005). Plant individuals as such are immobile, but the migration of vegetation occurs as seeds (e.g., Cain et al. 2000) carried by animals, wind, and human traffic. The southern species are likely to displace some arctic species, whose northward shifts are hindered by the Arctic Ocean (ACIA 2005). Therefore, unexpected chances may be expected in the vegetation.

While the temperature and its direct consequences on plants is the major force in plant adaptations to future climate, some other abiotic (and biotic) factors may also control the composition of the vegetation. Although the climatic change as such does not affect light conditions in the environment, the light climate experienced by the plants, however, changes in parallel as the vegetation migrates towards North. The plants of southern origin, therefore, have to adapt to changes in duration, quantity and quality of light. The daylength at the equator is continuously around 12 h throughout the year, while oscillation of the annual daylength increases towards the poles. Consequently, from the Polar Circle (66.5°N) northward polar night and summer exist, during which no sunrise and sunset occurs, respectively. Irrespective of the fact that the sun is above horizon continuously during the polar summer, the light environment changes toward the pole, where the solar height angle is the lowest. The phenomenon has two important consequences for plants. First, as the length of the path of solar radiation to the Earth's surface increases, the amount of photosynthetically active radiation (PAR) decreases. Second, for the same reason, light quality changes, since the lower wavelengths of the solar beam are scattered off more effectively as shown in Fig. 1. Light climate of the northern areas is well-documented elsewhere (Nilsen 1985).

2 Growth Responses at Changed Daylength

Plants are classified as long-day, short-day and day-neutral plants. These classifications concern especially the light requirement for flowering. Therefore, although climate is warming, short-day plants, such as rice cannot be cultivated in the Arctic areas. The flowering responses to daylength are well-documented in text books (e.g., Taiz and Zeiger 2006).

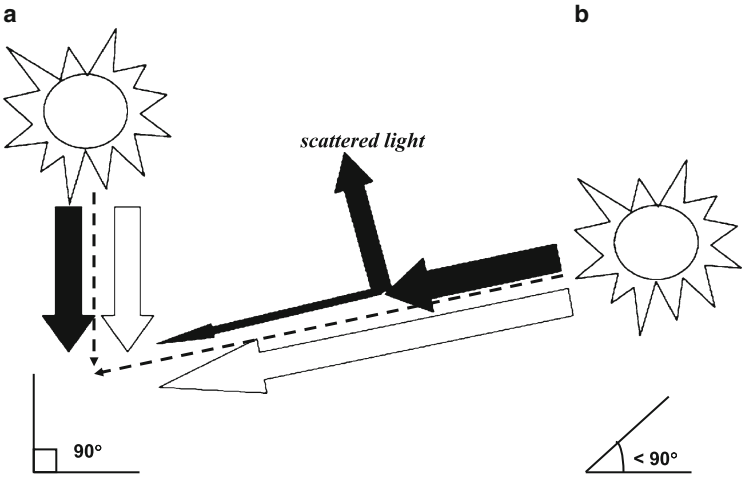

Fig. 1 Schematic illustration of the effect of solar elevation on the light quality reaching the earth's surface. Under high elevation (**a**) both the short (*black arrow*) and the long (*white arrow*) wavelengths of PAR reach the earth equally. Under low elevations (**b**) the relative proportion of the shorter wavelengths decreases due to scattering. The dashed arrows indicate the length of light paths

Daylength is the key factor, which determines the annual growth rhythm of a perennial plant. It also provides a signal for the correct timing between growth and dormant stages. Local provenances, i.e., ecotypes, are adapted to a local photoperiod (Vaartaja 1954, 1957), which ceases growth and initiates winter hardening processes (e.g., Heide 1974; Thomas and Vince-Prue 1997; Repo et al. 2000; Junttila 2007). Critical daylength is the shortest period during which elongation growth occurs, and it varies from 22 to 14–15 h between northern and southern ecotypes, respectively (Junttila 2007). For example, at 70°N latitude, the 22 h daylength occurs at the end of July. Indeed, a critical nightlength rather than daylength is also used as it refers better to the dark period. In practice, the more southern the ecotype is concerned, the longer the critical night, which is needed to trigger the arrest of growth and the onset of hardening process. In turn, these processes are induced by shorter nights (or longer days) in the northern ecotypes. For example, the experimentally determined critical night length decreases from 6 h to 3 h in silver birch ecotypes from 60°N and 67°N latitudes, respectively (Viherä-Aarnio et al. 2006). Because of the adaptation to longer night lengths in summer, a risk of delayed preparation for winter exists if a southern ecotype or species migrates towards the shorter nights of the north.

Dependence on photoperiod varies between species. Northern deciduous trees (e.g., *Betula* sp., *Populus* sp., *Salix* sp.) exhibit free growth pattern, according to which they continue shoot elongation growth until the critical daylength is exceeded (e.g., Junttila 2007). The cessation of growth is followed by bud dormancy and the development of frost hardiness. Species belonging to a predetermined growth pattern, such as Scots pine (*Pinus sylvestris)*, elongate in early summer when an interaction between ambient temperatures and the number of stem units produced in

the previous year's apical bud is given. Therefore, critical daylength does not determine the timing of growth cessation of species characterized by the predetermined growth. However, elongation of growth is reduced, if it occurs under shorter photoperiod than the ecotype has been adapted to, and oppositely longer photoperiod may even increase the elongation (Vaartaja 1954). In addition, Scots pine is able to develop marked frost hardiness only in response to shortening days at +20°C temperature (Taulavuori et al. 2000).

Latitudinal shift towards the north, obviously brings about competition between southern and northern populations, and even competition between species. For example, the growth response of southern plants may benefit from the longer days at northern high latitudes as shown by Vaartaja's (1954) ecotype experiments. However, if cessation of growth or development of frost hardiness in species or ecotypes is tightly controlled by photoperiod, they may suffer from delayed preparation for winter. However, certain plant genera from temperate climate (i.e., *Malus* sp., *Pyrus* sp.) may benefit from the shift of vegetation zones since their induction of dormancy is not controlled by photoperiod, but only by a drop in temperatures (Heide and Prestrud 2005).These species could extend their growing season compared to the species, which cease their growth in response to extending nights.

3 Growth Responses at Changed Light Quality

3.1 Responses to the Ratio of Red (R) to Far-Red (FR) Light (R/FR)

The midday R/FR ratio, i.e., ratio between red (660±5 nm) and far-red (730± 5 nm) light, is reported to be around 1.15–1.2 (Warrington et al. 1988; Smith 1994). The ratio decreases in the shade since the shorter red wavelengths of the light spectrum cannot penetrate through the foliage as effectively as the longer far-red wavelengths. Shade plants are adapted to a low R/FR (and low irradiance) environment, whereas shade-avoiding species strive for light by stem elongation. Smith and Whitelam (1997) described the shade avoidance syndrome by listing responses to shade in several physiological processes. These include reduced (or retarded) seed germination, leaf development, leaf thickness, leaf area, chloroplast development, chlorophyll synthesis, seed set, and branching. The shade avoidance performance also comprises increased extension growth, petiole and leaf extension, apical dominance, and flowering.

In addition to the photoperiodic control, the growth cessation of trees may be regulated by changes in light quality. There is a distinct clinal increase in requirement for FR light with increasing latitude of origin (Håbjørg 1972; Junttila and Kaurin 1985; Clapham et al. 1998; Mølmann et al. 2006). The northern ecotypes require higher intensity of FR to maintain growth than southern ecotypes, and the response of bud set to R increases with the northern latitude of origin

(Mølmann et al. 2006). In other words, northern populations require more FR to maintain growth compared to southern populations. This is in line with their adaptation to a lower R/FR ratio. The southern ecotypes, in turn, have adapted to a higher R/FR ratio and they need higher intensity of R to cease growth. Once shifted to far north along with climatic envelopes, it is possible that the slightly lower R/FR ratio may not be adequate to elicit a cessation of growth in the plants. However, response to changing R/FR ratios varies between and within species (Alokam et al. 2002; Vandenbussche et al. 2005; von Wettberg and Schmitt 2005). The shade avoiders typically consist of competitive and ruderal herbs, and pioneer tree species (Smith 1982 and references therein). Obviously, the northern R/FR ratio favors the shade tolerating or facultative shade plants.

Due to lower solar elevations, the R/FR ratio decreases slightly towards North being around 1.1 at 70°N latitude during summer solstice (Nilsen 1985). Because of the same reason, the ratio also decreases during sunset, being 0.9 when the sun reaches the horizon (Fig. 2). Thus, the light environment in the evening hours resembles conditions in the shade, since it is generally accepted that the R/FR ratio is a reliable index of the degree of shading (Kozlowski et al. 1991). Nilsen (1985) has also demonstrated reduced R/FR during summer solstice at Northern latitudes, whereas latitudes below the Polar Circle are characterized by a twilight or dark period. He raises an interesting question about a possible role of the reduced R/FR ratio in controlling the cessation of growth in the Arctic. In Spitsbergen (78°N), the midnight sun extends until August 22. Before that, the leaves have already turned yellow or plants have lost the leaves. The change in light quality, therefore, offers an excellent alternative for a seasonal timer in such places as the Arctic.

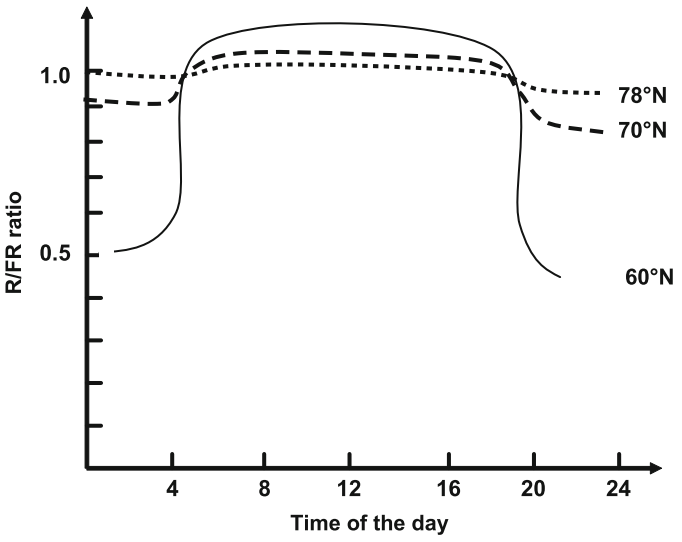

Fig. 2 Diurnal changes in R/ FR ratio (R = 660 nm; FR = 730 nm) at different latitudes in the time of summer solstice (redrawn after Nilsen 1985)

Mølmann et al. (2006) also proposed that the monitoring of light intensity at defined wavelengths could provide an alternative to the time-keeping of photoperiodism. However, the lowered R/FR ratio might be expected to act as a signal in the shade, indeed promoting growth (Junttila and Kaurin 1990; Clapham et al. 1998; de la Rosa et al. 1998; Aphalo and Lehto 2001; Mølmann et al. 2006).

3.2 Responses to Blue Light

Considering Nilsen's (1985) suggestion that a lowered F/FR ratio could cease the growth of plants in Spitsbergen, and that monitoring of "defined wavelengths" (Mølmann et al. 2006) could serve as an alternative time-keeping system, leads to the proposal that the increase in the relative level of blue wavelengths (B = 400–500 nm) is the decisive factor explaining the behavior of plants. As the solar angle diminishes, blue light (B) scatters effectively from the solar beam. However, a substantial amount of the diffused B is present during the "night-hours" of the Arctic summer. Figure 3 indicates that during days before summer solstice the R/FR ratio at 69°N latitude decreases from 1.2 to 0.5 for day and night, respectively. The night time ratio reported by Nilsen (1985) is higher (around 0.95) and explained by the fact that the sun remained behind the mountains during night. Nevertheless, the relative amount of B ratio (expressed as B/R) increased at this time from 0.85 to 1.3 for day and night, respectively.

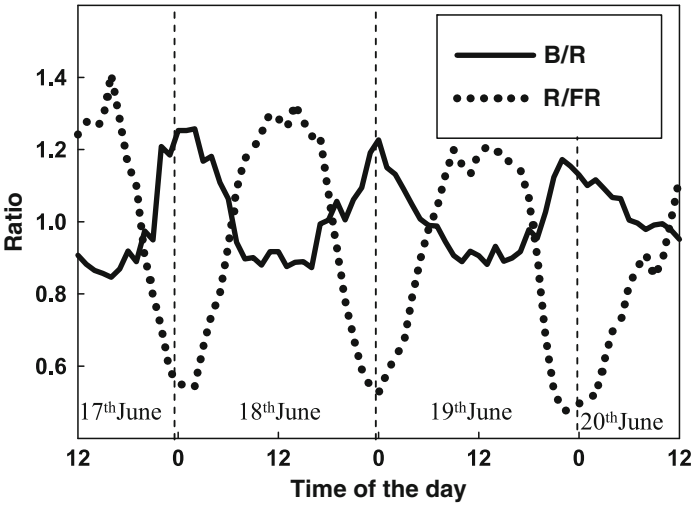

Fig. 3 An example of diurnal changes in ratios between blue and red (B/R) wavelengths, and red and far-red (R/FR) wavelengths at 69°N latitude (Kilpisjärvi, Finland) on 17th, 18th and 19th June 2008. The figure indicates that the relative amount of diffuse blue is high at "night" hours when the relative proportion of red light decreases

B as well as a high R/FR ratio inhibits stem elongation. Although responses to low R/FR ratio are mediated by the phytochrome family (Smith 1994), the signal of B is captured by both phytochrome and cryptochrome pigments. Fernbach and Mohr (1990) illuminated Scots pine seedlings with specific blue and red light, but only their parallel illumination with both wavelengths reduced the hypocotyl growth. According to the current knowledge, the inhibition of stem elongation occurs at least in two phases. In the first phase, B acts through the cryptochromes and in the second phase, R acts through the phytochrome (Parks et al. 1998; Folta and Spalding 2001; Cashmore 2006). In the phytochrome-mediated classical shade avoidance response, plants allocate more of their resources to the growth extension (Taiz and Zeiger 2006). The inhibited elongation regulated by B is a photomorphogenetic response, which closely resembles the phototropism reactions.

Artificial removal of the B region of the spectrum received by Scots pine seedlings improved their elongation significantly in the sub-arctic, while no such response occurred at the northern boreal latitude (Taulavuori et al. 2005a). Morphological data suggest that the response is not related to etiolation (Sarala et al. 2007). The sub-arctic sun has low elevation for several hours per day, and during this time the relative proportion of B increases due to a high amount of scattered and diffused B (Fig. 3). In the northern boreal site, the day ends towards the twilight within a relatively rapid transition from the daylight period. This (i.e., the lack of long-lasting B rich period) is the obvious reason why the response was found only at the high latitudes.

From the "climatic envelope" concept, it follows that the plants that colonize the Arctic areas will receive more indirect B during low solar elevations at the end of days of the growing season, compared to that they have been adapted to. The expected consequence is a reduced elongation.

3.3 Responses to UV-Radiation

The stratospheric ozone absorbs UV-radiation (UV_{ab} = 280–400 nm) from the sun. Ozone depletion has taken place in the southern polar region, causing the so-called "Antarctic ozone hole." A similar, though less severe, depletion of ozone is observed in the northern polar region of the Arctic. The UV-dose may be especially high in spring as the reflectance from snow may increase the dose by over 50% (ACIA 2005). UV-B radiation (280–315 nm) especially damages DNA through, e.g., lethal or mutagenic pathways. Many Arctic species are fortunately preadapted to relatively high levels of UV-B radiation, and exhibit various mechanisms of adaptations (thick cell walls and cuticles, waxes, hairs on leaves, UV absorbing chemical compounds, etc.) (ACIA 2005).

Also, plant seasonality may be disturbed due to a long-term exposure to UV-radiation. UV-radiation may hamper the development of plant frost hardiness (Beerling et al. 2001; Taulavuori et al. 2005b), obviously due to trade-off

production between screening and cryoprotective compounds (Taulavuori et al. 2005b). However, different responses are also reported (Dunning et al. 1994; Teklemariam and Blake 2004), and it is thus possible that responses vary between species. If the hypothesized trade-off between cryoprotection and pigmentation exists, northern species and ecotypes could therefore be expected to be more competitive than southern species, as the former have adapted to a wider range in frost hardiness.

4 Growth Responses at Changed Light Quantity

Light quantity may be considered from two perspectives. Fluence rate is the irradiance received by a plant at a given moment. Total (daily or annual) rate indicates accumulated irradiance over a given time. Light energy and fluence rate decrease towards North because of the lower solar angle. However, the continuous day of polar summer compensates the decrease in both parameters, and thus, the accumulated PAR does not differ between the intermediate and high latitudes (Pinker and Laszlo 1992). The global radiation (i.e., direct and diffuse) instead distinctly decreases towards the poles due to decreasing solar angle. Based on the data collected and averaged by the Finnish Meteorological Institute during years 1971–2000 separately for each month (FMI 2009), we calculated ($r^2 = 0.9$) that the averaged global radiation in June and July decreases approximately by 20% along a 1,000 km distance from 60.1°N (Helsinki) to 69.45 (Utsjoki, Kevo). Körner (2003) reported that mean photon flux density (PFD) from the daily accumulated fluxes in the Alps region is around 750 μmol $m^2 s^{-1}$, while in the Scandinavian Polar circle it is around 415 μmol $m^2 s^{-1}$. An illustration of the diurnal PFD at different latitudes is shown in Fig. 4.

If vegetation migrates towards north, where less irradiance is available, it is understandable that some species cannot receive enough light for saturation of photosynthesis. The available PFD may, thus somewhat, limit growth of some species, and this phenomenon may modify the composition of the vegetation. For example, the light saturation point of Scots pine (*P. sylvestris*) is reported to be around 800 μmol $m^2 s^{-1}$ (Troeng and Linder 1982), while photosynthesis of Norway spruce (*Picea abies*) is saturated only at a 30% level of that (Kellomäki 2005), which represents approximately for 330 μmol $m^2 s^{-1}$. Figure 4 demonstrates that Scots pine does not reach the light saturation level during midday at the Polar circle (66.5 N), while photosynthesis of Norway spruce is light saturated for 16 h. At the prevailing climate, Norway spruce remains at lower latitudinal (and altitudinal) elevations in the Arctic treeline compared to Scots pine due to a weaker seed production. However, once the temperature barrier is removed as a consequence of the warming climate, the northern light quantity should favor Norway spruce in competition with Scots pine. According to the table compiled by Larcher (2003), it may be concluded that mosses also benefit at the expense of lichens, for example, under lower light quantities.

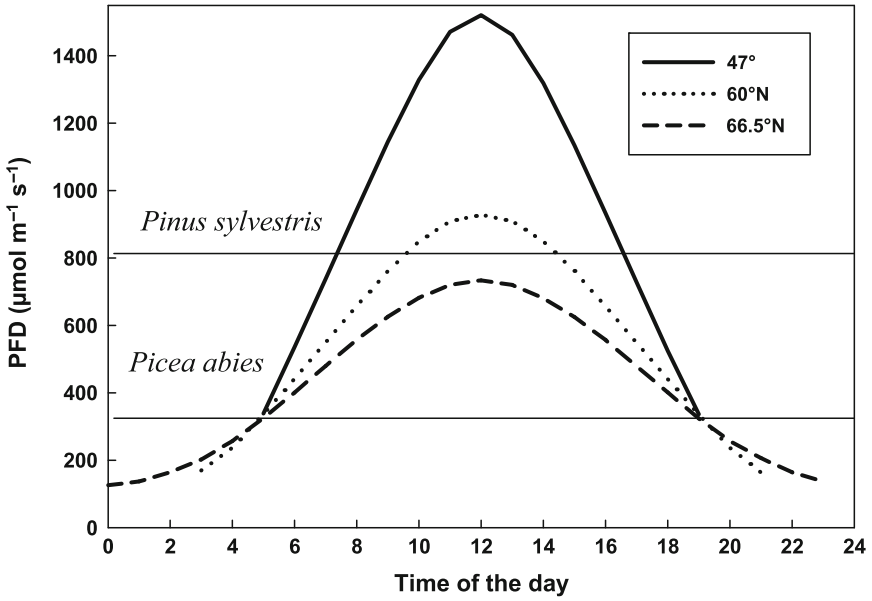

Fig. 4 Rough estimations for the diurnal distributions of PFD (photon flux density) at 47, 60 and 66.5°N latitudes on the 15th June. The PFD values are derived as follows: (1) Midday solar elevation and the theoretical midday solar power (W m^{-2}) were taken from Lammi's (2001) daylength calculator for different latitudes, and regression between them was calculated. (2) Using the resulting regression equation, solar power was calculated for solar elevations of full hours at each of the given latitudes. (3) The solar power values were converted to full sun plus sky PFD values by multiplying with 2 (Tang 1997). (4) As the actual cloud formation reduces PFD by 75% or more (Tang 1997), the PFD data were compared to daily means in the Alps (47°N, 750 µ mol $m^{-1}s^{-1}$) and at the Scandinavian Polar Circle (66.5°N, 415 µmol $m^{-1}s^{-1}$) (Körner 2003). The comparison indicated that the measured values were 65% and 42% of theoretical values for the Alps and the Polar Circle, respectively. Reduction from theoretical irradiance at 60°N was calculated from these values, resulting in a 48% reduction. (5) Finally, the PDF values of full hours were corrected against the above reductions. Note that only full hours are considered

5 Conclusions

It is obvious that competition will occur among the vegetation, once species and ecotypes migrate towards north. In Scandinavia, the final boundary is the Arctic Ocean, which prevents the northward shifts (ACIA 2005). There will be a competition between species and ecotypes over better adaptation to the Arctic light environment. Adaptation concerns three major factors: (1) correct seasonality controlled by photoperiod, (2) light saturation (i.e., adequate resource acquisition) controlled by light quantity and (3) height growth (and consequent light competition) controlled by light quality. We did not discuss about the genetic aspects as the main focus in plant responses to a changed light environment.

Savolainen et al. (2007) proposed that current migration of trees is slow relative to climate change, which may hamper an evolutionary response to the climate change. This holds true, if only "natural" (wind, animals) dispersal is under question. However, increasing tourism and the forest owners, whose anticipation is to maximize forest productivity also in the future climate, may speed up the migration rates. Therefore, it is important to test the following hypotheses experimentally or through modeling ways, both at the plant community and population level. For example, it may be questioned if the populations are locally adapted having always the best fitness in their local site, or if they have higher fitness in the south, but the highest relative fitness at their original site (Kawecki and Ebert 2004; Savolainen et al. 2007). Thus, if the plant responses to changing environment were predictable, it is possible to conserve the vegetation of Arctic areas, e.g., through political and management decisions. This could provide time for the northern ecosystems to adapt to the future environment and competition over there.

We wish to point out the following:

- Preparation for winter of southern species or ecotypes may be delayed in the northern photoperiod, although some species independent of photocontrol may even benefit from it
- Lower R/FR in the north may retard growth cessation of southern ecotypes of certain species, and the ratio may favor facultative shade species
- Southern ecotypes or species may reduce elongation in the northern environment enriched by blue light during low solar elevations
- UV-radiation tolerating species survive generally better, but the frost hardening process of certain species may be impaired due to enhanced UV-radiation
- Species with lower light saturation points may benefit at the expense of species with higher light saturation points

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Initial Events Associated with Virus PBCV-1 Infection of *Chlorella* NC64A

Gerhard Thiel, Anna Moroni, David Dunigan, and James L. VanEtten

Contents

Abstract *Chlorella* viruses (or chloroviruses) are very large, plaque-forming viruses. The viruses are multilayered structures containing a large double-stranded DNA genome, a lipid bilayered membrane, and an outer icosahedral capsid shell. The viruses replicate in certain isolates of the coccal green alga, *Chlorella*. Sequence analysis of the 330-kbp genome of *Paramecium bursaria Chlorella* virus 1 (PBCV-1), the prototype of the virus family *Phycodnaviridae*, reveals <365 protein-encoding genes and 11 tRNA genes. Products of about 40% of these genes resemble proteins of known function, including many that are unexpected for a virus. Among these is a virus-encoded protein, called Kcv, which forms a functional K^+ channel. This chapter focuses on the initial steps in virus infection and provides a plausible role for the function of the viral K^+ channel in lowering the turgor pressure of the host. This step appears to be a prerequisite for delivery of the viral genome into the host.

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1 Introduction

Chlorella viruses belong to the family *Phycodnaviridae*, genus *Chlorovirus*. They are large (190 nm in diameter), icosahedral, plaque-forming viruses with linear dsDNA genomes (Van Etten 2003; Yamada et al. 2006; Wilson et al. 2009). The type member of the genus is *Paramecium bursaria Chlorella* virus (PBCV-1); it has a genome of 331 kb that contains at least 365 protein-encoding genes and a polycistronic gene that encodes 11 tRNAs. Approximately 40% of its predicted gene products resemble proteins of known function, many of which are unexpected for a virus. The PBCV-1 virion has a glycoprotein shell that surrounds a lipid bilayered membrane.

Chlorella viruses infect certain freshwater, unicellular, eukaryotic *Chlorella*-like green algae, which normally exist as endosymbionts in protists. However, they are grown in the laboratory as axenic cultures. The addition of PBCV-1 to its host, *Chlorella* NC64A, leads to the following program of events: (1) virus attachment to the cell wall is host-specific and probably occurs at a unique virus vertex (Onimatsu et al. 2006; Cherrier et al. manuscript submitted for publication), followed by wall degradation at the point of attachment (Meints et al. 1984); (2) rapid host membrane depolarization (Frohns et al. 2006), potassium ion release (Neupärtl et al. 2007) and altered secondary active transport of solutes (Agarkova et al. 2008); (3) host nuclear DNA degradation beginning at 3–5 min postinfection (p.i.) (Agarkova et al. 2006); (4) early viral transcripts beginning to appear within 5–10 min p.i. (Schuster et al. 1986; Kawasaki et al. 2004); (5) virus DNA replication beginning at 60–90 min p.i. (Van Etten et al. 1984); (6) late virus transcription beginning at 60–90 min p.i. (Schuster et al. 1986), and (7) viral-induced lysis and particle release occurring at 6–8 h p.i. (Van Etten et al. 1983; Meints et al. 1984).

Many aspects of the *Chlorella* virus/host system have been extensively covered in several recent reviews (Van Etten 2003; Yamada et al. 2006; Dunigan et al. 2006; Wilson et al. 2009). This chapter focuses on the initial events of infection. It is motivated by the finding that *Chlorella* viruses code for K^+ channel proteins and we present a hypothesis on how the activity of these channels is relevant to the entry of the virus DNA into the host cell.

2 Infection

Virus PBCV-1 infection of *Chlorella* NC64A cells is an excellent example of how a virus is able to rapidly take over the control of its host cell. The first step in PBCV-1 infection is attachment to the host cell wall (Meints et al. 1984). Presumably, a virion protein(s) interacts specifically and strongly with some component(s) in the host cell wall. This specificity determines the narrow host range of the *Chlorella* viruses and the strength assures that attachment withstands subsequent dissociation by environmental stresses.

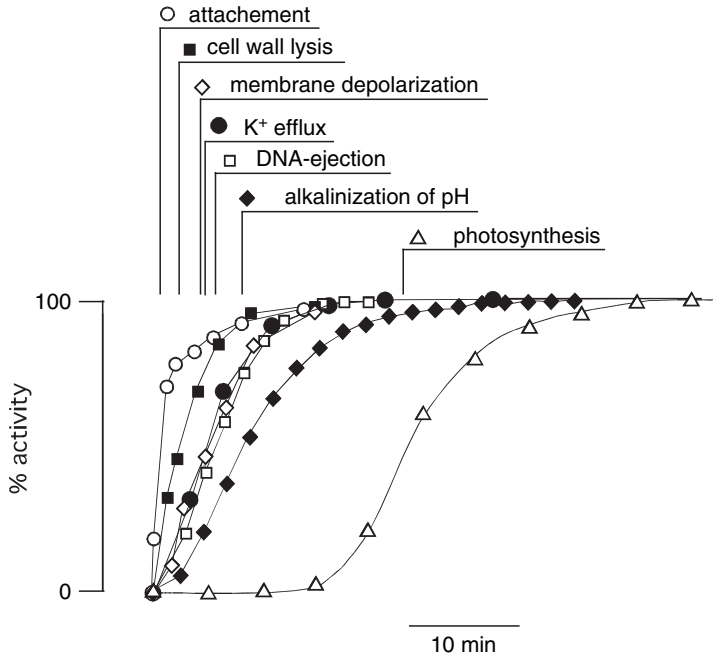

Fig. 1 Mean time course of distinct early events during infection of *Chlorella* NC64A cells by virus PBCV-1. The half times ($t_{1/2}$) of attachment, cell wall lysis membrane depolarization, K^+ efflux, DNA ejection, cytosolic alkalinization and inhibition of photosynthesis are indicated. Data from: depolarization, Frohns et al. 2006; attachment/ cell wall lysis/ K^+ efflux/ DNA ejection: Neupärtl et al. 2007, alkalinization, Agarkova et al. 2008; photosynthesis, Seaton et al. 1995

The attachment process is rapid. Analysis of electron-microscopic images of *Chlorella* NC64A cells fixed at various times after mixing the host cells with the virus indicate that attachment is complete with a half time ($t_{1/2}$) of ca. 1 min (Fig. 1). This value describes the attachment of a population of particles, although individual virions may only require a few seconds.

Virus attachment to the host cell wall is not random; attachment always occurs at a virus vertex (Meints et al. 1984). Recent experimental results indicate that not all 12 virus vertices are identical and that attachment probably occurs at a unique tail-containing vertex (Onimatsu et al. 2006; Cherrier et al. manuscript submitted for publication). The PBCV-1 protein A140/145R is preferentially localized at this unique vertex and is essential for binding to the host cell wall. The positioning of the virus to this unique vertex could be brought about by external fibers on the virion (Van Etten et al. 1991; Cherrier et al. manuscript submitted for publication).

The idea of a polarized and function-specific vertex is consistent with prevailing models on the organization of other tail-containing, icosahedral bacterial viruses (phage) that enter the cell and leave the capsid at the surface. These phages, which

have the same geometric capsid architecture as PBCV-1, have a portal protein complex attached to a tail at one vertex; typically, this vertex provides the pathway for both genome encapsidation and genome delivery (Cerritelli et al. 2003; Lebedev et al. 2007). However, the huge amoeba-infecting Mimivirus, which has a similar icosahedral morphology, including an internal membrane, and shares a common evolutionary ancestor with the *Chlorella* viruses, has two portals, one for DNA packaging and another for DNA ejection (Zauberman et al. 2008).

Thus, PBCV-1 infects *Chlorella* NC64A cells in a bacteriophage like manner, meaning that it ejects its genome and certain proteins into the host cell leaving an empty capsid attached to the host (Meints et al. 1984). From a mechanistic point of view, this is a challenging process, because the viral genome has to pass several barriers namely: (1) the viral membrane, (2) the capsid, (3) the cell wall of the host and (4) the plasma membrane of the host.

This entire process occurs in a way that the integrity of the host plasma membrane, i.e., the barrier for unspecific ion fluxes, is not compromised.

After attachment, the next step in the process is localized digestion of the host cell wall (Meints et al. 1984; Neupärtl et al. 2007). The virion particles include one or more enzymes that are secreted after attachment to degrade the host cell wall (Yamada et al. 2006). These hydrolytic enzymes rapidly digest a hole into the cell wall, with a $t_{1/2}$ of ca. 2.5 min (Fig. 1). The first holes in the host cell wall are observed after 1 min p.i.

3 Transfer of DNA from the Virus Particle into the Host Cell

After attachment and digestion of the host cell wall, the virus ejects its DNA, and also probably several proteins, into the host (Fig. 1). Neither the energetics for the injection of viral DNA into the host nor the pathway for the transfer of virus DNA/proteins into the host cells is completely understood. The virus particle has a glycoprotein capsid surrounding an internal membrane; the DNA genome and probably several proteins are contained inside this membrane. Proteomic analyses of PBCV-1 particles indicate that the virion contains more than 100 different, viral encoded proteins (Dunigan et al. manuscript in preparation), only a few of which probably serve a structural role. An important question is: how does the virus transfer its DNA and some proteins across its own membrane and across the membrane of the host? Experimental evidence indicates that this transfer occurs without compromising the integrity of the host plasma membrane; measurements of the cytoplasmic pH (Agarkova et al. 2008) and photosynthetic activity (Van Etten et al. 1983; Seaton et al. 1995) of the host cells indicate that virus infection results in alkalinization of the host cytoplasm ($t_{1/2} = 8$ min) and a decrease in photosynthesis ($t_{1/2} = 24$ min) after a short lag time (Fig. 1). The external medium of the *Chlorella* cells is slightly acidic, pH ~5.5. If DNA transfer, which occurs with a $t_{1/2}$ of less than 6 min. p.i. (Fig. 1), were corrupting the integrity of the host cell plasma membrane, acidification of the cytoplasm should occur in the same time frame.

The experimental results indicate that DNA ejection is an interplay between virus and host. The images shown by Meints et al. (1984) indicate that PBCV-1 attaches to and digests isolated cell walls of the host; however, DNA is not released (Meints et al. 1984). Hence, the virus requires a signal from the host to eject DNA. What is the nature of this signal? The answer to this question is unknown. However, we can make some educated guesses based on simple knowledge of plant physiology. The host cell *Chlorella* NC64A is a freshwater alga. Freshwater algal cells have a high turgor pressure that is roughly in the order of 1 MPa (Kim et al. 2006). *Chlorella* NC64A cells have an intracellular K^+ concentration of about 100 mM (Neupärtl et al. 2007), which resembles other freshwater algae; thus, *Chlorella* NC64A has the same high turgor pressure as other freshwater algae.

The formation of a hole in the host cell wall, such as those produced by PBCV-1, is equivalent to a hole in the outer tube of a bicycle tire. If the hole is sufficiently large and the inner pressure is high enough, the inner tube will bulge. Whether the host plasma membrane bulges out of a hole in the cell wall is determined by the turgor pressure pushing it, the geometry of the hole (i.e., the area over which the pressure is exerted), and the tensile forces in the membrane. As an approximation, this relationship is given by (1)

$$\cos \alpha = Pr/2\sigma. \tag{1}$$

This equation describes the movement of a liquid in a capillary; α is the contact angle (given in degree) of the membrane bulge with the cell wall, r the radius of the hole, σ the membrane tension, and P the turgor pressure of the cell. To calculate the contact angle[1] and, thus, the geometry of the bulge, we measured the mean diameter of the cell wall hole produced by the virus in 65 electron micrographs, e.g., (Figs. 2 and 3). A mean radius of 53 ± 4 nm was obtained. The tension of cellular plasma membranes is generally low. Careful estimates of this parameter in neuron cells produced values of 0.03–0.04 mN m^{-1} (Dai et al. 1998). Since the biophysical properties of membranes from animals and plants are similar (Morris and Homann 2001), we used this value in the following calculations. Figure 2a shows a plot of the estimated contact angle as a function of the radius of the virus generated hole in the cell wall. The data were calculated by assuming a membrane tension of 0.03 mN m^{-1} and a turgor pressure of 1 MPa. The latter value is a good estimate of the turgor pressure in freshwater algae. The plot in Fig. 2a, which is only a crude estimate of reality, nonetheless indicates that the size of the hole generated by the virus is sufficient to generate near maximal bulging of the membrane. If we superimpose the geometry of such a bulge on an electron micrograph (Fig. 2b), the membrane is pushed towards the viral particle.

[1]The contact angle α describes the orientation of the membrane plane with respect to the inner lining of the small hole in the cell wall. An angle of 0^o means that the plane of the membrane is quasi parallel to the wall; e.g. the membrane reveals an omega shape bulge. If at the other extreme the angle is 90^o the membrane is perpendicular to the wall; the membrane remains flat in the plane without any budging out.

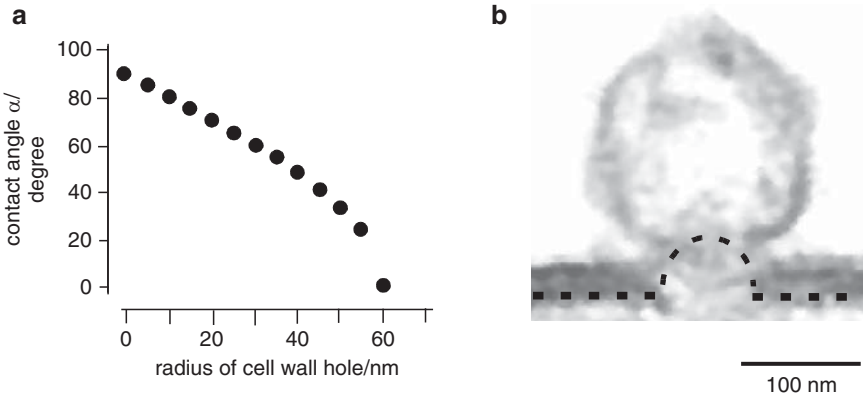

Fig. 2 The production of a hole in the host cell wall results in host membrane bulging. (a) Calculated contact angle of budding membrane as a function of radius of the hole in the cell wall. (b) Cartoon of host cell membrane (*dashed line*) budding through the cell wall hole towards the virus particle with a contact angle α of 0°

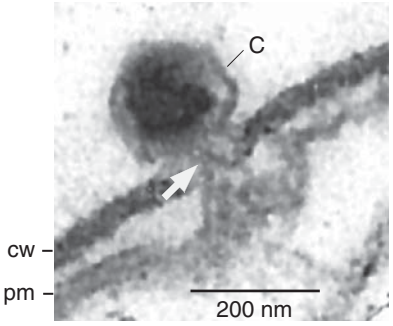

Fig. 3 The host cell membrane is intimately connected with the virus particle. An image of PBCV-1 infecting *Chlorella* NC64A. The virus (c) has digested a hole into the host cell wall (cw). At the site of infection the plasma membrane (pm) has retracted from the cell wall, probably due to an artifact of the fixation. However, where virus/cell contact occurs the plasma membrane of the host is intimately connected with the virus particle (*arrow*)

The bulging of the host cell membrane alone is insufficient to explain fusion of host and viral membranes. Notably, the viral membrane is inside the capsid. However, analysis of the PBCV-1 capsid structure by atomic force microscopy indicates that a central protein in the virion vertex is pushed open by applying external mechanical force (Kuznetsov et al. 2005). In the context of our model, it is reasonable to assume that the host cell membrane pushes this "valve like structure" open. However, the virion would have to undergo an additional structural change to increase the size of the virus hole before the host cell membrane could enter the interior of the virion. It remains to be seen how this interpretation fits together with

new structural information on PBCV-1 that indicates the virion has a thin 250 Å long and 50 Å wide tail at one vertex (Cherrier et al. submitted for publication).

Membranes have to be brought into close physical contact to fuse. This is generally achieved in cells by SNARE proteins (Jahn and Scheller 2006) or specific fusion proteins in viruses (Harrison 2008). The *Influenza A virus* for example contains a specific protein, hemagglutinin, which forces the viral membrane close to the host membrane of the endosomes leading to membrane fusion (Chernomordik et al. 1999). In the PBCV-1/*Chlorella* system, viral and host membrane fusion might not require a specific protein(s). The high turgor pressure in plants might force the host membrane towards the viral membrane, leading to fusion. This hypothesis is plausible because it is well known that physical pressure and membrane tension can catalyze membrane fusion (Shillcock and Lipowsky 2008; Thiel et al. 2000).

Currently, this description of virus/host membrane fusion is hypothetical, but nonetheless, consistent with some experimental observations: (1) the hypothesis predicts that reduction in host turgor pressure should inhibit PBCV-1 infection. When *Chlorella* NC64A cells are pre-incubated with 400 mM sorbitol to reduce their turgor pressure, they are not infected by PBCV-1 (Hampe and Thiel unpublished results). (2) Electron micrographs of infected *Chlorella* NC64A indicate that host cells are often slightly plasmolyzed, i.e., there is a retraction of the plasma membrane from the walls (Fig. 3). However, the membrane is always attached to the wall at the point of virus entry. These images indicate that the host plasma membrane is in intimate contact with the virus.

Additional evidence for membrane fusion is also circumstantial. PBCV-1 codes for a K^+ channel protein, named Kcv (Plugge et al. 2000). Similar, but not identical, K^+ channel proteins are also coded by related *Chlorella* viruses (Balss et al. 2008; Gazzarrini et al. 2009; Kang et al. 2004b; Gazzarrini et al. 2006), which suggest that the gene product is important for viral replication. In the case of PBCV-1, the channel protein is synthesized as a late gene in the host (Kang et al. 2004a); late virus proteins are often packaged in nascent viruses. We have suggested that the viral channel is present in the virus internal membrane (Frohns et al. 2006). This suggestion leads to the prediction that fusion of the viral K^+ channel containing membrane with the host membrane should result in depolarization of the host plasma membrane potential.

To understand the consequences of fusion of the viral membrane, containing one or more Kcv channels, with the host plasma membrane, a few basic transport properties of the host cell as well as the unitary conductance of the viral K^+ channel need to be considered. Single channel conductance of the Kcv channel is known; the Kcv protein has been produced as a recombinant protein and reconstituted into planar lipid bilayers (Pagliuca et al. 2007); furthermore, it was expressed in the plasma membrane of *Xenopus* oocytes (Abenavoli et al. manuscript submitted for publication). The resulting Kcv single channel activity indicates that it has a very high unitary conductance. Conductance is about 130 pS in a solution of 100 mM K^+ (Pagliuca et al. 2007). In contrast, a typical plasma membrane with K^+ channels, such as the *Arabidopsis* K^+ channel KAT1, has a unitary conductance of 10 pS (Thiel and Wolf 1997).

While the viral K^+ channel has very high conductance, the resting conductance of the plasma membrane of freshwater algae, such as *Chlorella*, is generally low. In the well-studied green alga *Chara corallina*, the plasma membrane has a resting resistance of about 1 Ωm^2 (Beilby 1985). This high resistance occurs because most K^+ channels in the membrane are closed at rest. The resting resistance of the *Chlorella* NC64A plasma membrane is unknown, but is probably similar to *Chara corallina*. Measurements of *Chlorella* NC64A membrane voltage with fluorescent dyes indicate that increasing external K^+ concentration has no detectable effect on membrane voltage (Frohns et al. 2006). This result is consistent with a low membrane conductance at rest and a high resistance for K^+ transport.

To evaluate the result of viral and host cell membrane fusion, it is necessary to look closely at the electrical properties of the host. A plant cell membrane, such as *Chlorella*, can be represented by a simple equivalent circuit (Fig. 4; Thiel and Gradmann 1994). The membrane contains a H^+ ATPase and, in parallel, other passive conductances, such as channels and transporters. Each of the transporters has an inherent resistance and a battery voltage. The ATPase with the inherent resistance R_P can power the membrane to a voltage E_P, which can be as negative as

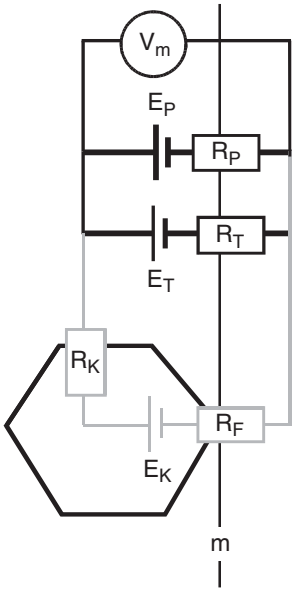

Fig. 4 Equivalent circuit of host cell membrane before and after virus infection. The membrane potential (V_m) of the host cell plasma membrane (m) at rest is generated by the parallel arrangement of active and passive transporters (*black lines*). The active transporter, the proton ATPase, has an inherent resistance R_P and creates a battery voltage E_P (ca. −400 mV). The parallel transporters (channels, carriers etc.) are summarized here with their resistance R_T and their equilibrium voltage E_T (< 0 mV). Because $R_P << R_T$, V_m is very negative at rest. During infection the electrical properties of the viral particle, which contains the K^+ channel with the resistance R_K, are connected (*gray lines*) via a low resistance fusion pore (R_F) with the membrane of the host. Because $R_K << R_P$ and R_T, V_m becomes dominated by E_K, i.e., depolarizes

−400 mV. The reversal voltages of all the remaining transporters (summarized as R_T,) are more positive. For simplicity, we assume that E_T is 0 mV. The prevailing membrane voltage (V_M) of a plant cell is given by these battery voltages and the relative resistances of the transporters R_P and R_T. Because the resistance of pump R_P in plants is much lower than R_T, the membrane voltage is highly negative. Note that some freshwater algae have voltages as negative as −350 mV.

For the aforementioned reasons, fusion of the virus membrane with the host membrane will electrically connect the virus membrane containing the Kcv channel R_K with that of the host via a low resistance, the fusion pore R_F. If we assume a typical membrane resistance in a fresh water alga of 1 Ωm^2, we can calculate the overall resistance of a *Chlorella* cell. These spherical algae have an average diameter of 4 μm, i.e., a surface area of $5 \times 10^{-11} m^2$. Hence, their electrical conductance is 200 pS. This value is well in the range of the unitary conductance of the viral channel. Assuming that the virion carries ≥1 channel proteins R_P and R_T become smaller than R_K. Consequently, the circuit voltage is dominated by the battery voltage (the Nernst potential, E_K) of the viral K^+ channel. As a consequence, the host cell membrane potential V_M must shift towards E_K. Because E_K is more positive than the plant resting membrane voltage, Kcv incorporation into the *Chlorella* membrane causes depolarization.

The aforementioned description of the electrical properties of the virus and the host predict that fusion of the two membranes results in depolarization of the host cell membrane. This prediction was tested by measuring the membrane voltage of *Chlorella* cells during virus infection (Frohns et al. 2006; Neupärtl et al. 2007). *Chlorella* cells are not suitable for conventional voltage recordings with microelectrodes, because they are small and have rigid cell walls. Therefore, fluorescent dyes were used to monitor changes in the membrane voltage. The data in Fig. 1 show that the experimental results agree with the prediction. Less than a minute after infecting *Chlorella* NC64A with PBCV-1, the dye fluorescence increases and reached half maximum ($t_{1/2}$) after 5 min p.i. (Fig. 1). This increase in fluorescence, which remains for several hours after infection (Frohns et al. 2006), is due to depolarization of the *Chlorella* membrane. The depolarization immediately follows the formation of the hole in the *Chlorella* cell wall during infection (Fig. 1). These results support the causal relationship between the bulging out of the host cell membrane and fusion with the viral membrane.

Several experimental results support the hypothesis that depolarization is induced by the viral K^+ channel. The strongest support comes from the observation that different *Chlorella* viruses encode K^+ channels with different pharmacological properties (Frohns et al. 2006). The PBCV-1 Kcv channel is Cs^+ insensitive, while the Kcv channel from virus NY-2A is Cs^+ sensitive. Consistent with the hypothesis that depolarization is due to the viral channels, depolarization is inhibited by Cs^+ when cells are infected with virus NY-2A. In contrast, Cs^+ only has a small effect on the depolarization evoked by virus PBCV-1 (Frohns et al. 2006).

Collectively, these data strongly support the hypothesis that the viral membrane and the host plasma membrane fuse very early during infection.

4 Energetics of DNA Ejection

Let us assume the viral membrane fuses with the host membrane and creates a sizable fusion pore between particle and host, which serves as the conduit for transferring the viral genome into the host. However, this scenario does not provide any energy, which is required for this process because the plant has an internal pressure of about 1 MPa. Hence, the virus has to eject DNA against this huge pressure. To explain this process, it is helpful to understand how bacteriophages solve the problem. Like PBCV-1, many phages have large dsDNA genomes and they have to eject DNA into highly turgerized bacteria cells (Molineux 2006; Grayson and Molineux 2007).

A property that PBCV-1 shares with certain bacteriophages is that they pack a large dsDNA genome into a small geometrically confined capsid. For example, phage λ has a 48.5 kbp dsDNA genome, which in its extended linear form is ∼16.5 μm long (Grayson et al. 2007). The entire DNA is packed into a capsid with an internal radius of 27.5 nm (Cordov et al. 2003); these dimensions translate into an ∼540-fold linear compression of the DNA molecule. This compression value is similar to all known dsDNA phages, which compress their genome at a density of about 500 mg ml^{-1} (Molineux 2006). The genomes of *Chlorella* viruses, which range from 295 to 360 kb (Landstein et al. 1995; Fitzgerald et al. 2007a,b), are packed into capsids with an inner diameter of about 800 Å. Assuming that the capsid is a sphere, we calculate a ratio of genome per volume. This ratio is 0.5 pb nm^{-3} in the case of phage λ and 0.15 pb nm^{-3} for PBCV-1. The fact that both numbers are the same order of magnitude and that the PBCV-1 capsid also packages other proteins implies that both viruses have a similar challenge for packing their DNA into a small volume.

Native DNA has a double helical structure with two sugar–phosphate chains on the outside that are joined by complementary base-pairs projecting into the helix-interior. In particular, the large size and the high negative charge of the DNA molecule relative to the surrounding solvent molecules and salt ions makes DNA packaging a challenging process. For this reason, many studies have been performed on DNA packing in phages; however, this process is still not fully understood.

Recent experiments and theoretical investigations indicate that the DNA in the small capsid generates enormous internal pressure in the particles ranging up to 5 MPa (Li et al. 2008; Molineux 2006; Grayson and Molineux 2007). This internal pressure inherent in the stored DNA is, at least in some phages, used as a driving force for the rapid ejection of DNA from the virus particle. Recent experiments monitored the ejection of DNA from phage λ in real time. The results indicate that the entire 48.5-kbp genome is expelled in ca.1.5 s without interruption, reaching a speed of 60 kbp s^{-1} (Grayson et al. 2007). The similarity between DNA packing in phages and the *Chlorella* viruses suggests that at least some of the energy for ejection of PBCV-1 DNA also derives from the energy inherent in DNA packing.

Even though this pressure driven mechanism of DNA ejection appears plausible, new observations indicate that it only explains part of the story. Though the λ and

T5 genomes are indeed fully ejected from their capsids into buffer, the situation is different when looking at ejection into host cells (Molineux 2006). In the in vivo situation, the pressure inside the host cell seems to prevent full ejection of the genome. Hence a push–pull mechanism occurs in some bacteriophages in which the initial part of the genome is pushed into the host by the pressure inherent in the compressed DNA and the remaining portion of the genome is pulled in by proteins inside the host (Molineux 2006). This type of genome transfer has been well studied in phage phi29, where the leading 60% of the DNA is pushed into the host in a pressure dependent manner while the remaining part requires energy to be pulled in (Gonzalez-Huici et al. 2004).

Other experimental studies on phages indicate that the pressure derived from the packing of the DNA is insufficient for transferring the entire genome into the host. Therefore, the internal pressure in the host cell must be reduced to allow complete ejection of the viral genome. Bacteriophages accomplish this by causing host cell membrane depolarization and a concomitant release of K^+ salts from the host (Boulanger and Letellier 1992; Labedan and Letellier 1981). The situation is similar to the *Chlorella* viruses. *Chlorella* membrane depolarization results in an ~50% loss of K^+ from the host in less than 5 min after infection (Fig. 1, Neupärtl et al. 2007). The release of K^+ probably results from activity of the viral Kcv channel. First, K^+ release occurs with the same kinetics as membrane depolarization. Second, viral K^+ channel blockers partially reduce K^+ release from the host cells (Neupärtl et al. 2007). The activity of the Kcv channel, however, is not sufficient to explain the entire scenario. To generate a driving force for K^+ efflux from the host cells, the membrane potential must be depolarized well positive of the K^+ equilibrium potential (Neupärtl et al. 2007; Thiel and Gradmann 1994). This situation is only possible, if the initial depolarization activates other channels in the host membrane. This hypothesis is reasonable because many plant cells contain anion channels, which can be activated indirectly via Ca^{2+} mediated membrane depolarization (Sanders et al. 2002).

5 Virus Infection Rapidly Alters the Physiology of the Host

The infecting virus is on a volume basis ca. 10^4 times smaller than the host cell. However, still it is able to rapidly commandeer major activities in the host cell. Within 5 min after infection, host DNA begins to be degraded by virus packaged DNA restriction endonucleases; a couple of minutes later, the first viral transcripts are detected in the host cell (Agarkova et al. 2006). About 10 min p.i., the host cells begin to shut down photosynthesis with a $t_{1/2}$ of about 24 min (Van Etten et al. 1983; Seaton et al. 1995). In the case of a related system with *Chlorella* Pbi cells and virus CVM-1, inhibition of photosynthesis is preceded by an active alteration of photosytem II (PSII). Due to the rapid expression of a viral gene, probably a de-epoxidase, PSII rapidly losses its efficiency to absorb light energy (Seaton et al. 1996). Because of the rapidity of all these processes, the virus must possess mechanisms that favor the activity of its replication over those of the host. Details

on how this take over of the host is achieved are unknown. However, virologists have suggested that the most effective way for a virus to achieve control is to modify one of the "master switches" in the host. Such a master switch could be any cellular process that affects many different metabolic and signaling pathways in the cell. This certainly happens when *Chlorella* cells are infected by PBCV-1. First, as mentioned above, the membrane voltage of the host collapses early during infection (Frohns et al. 2006). This collapse has consequences for the ionic milieu of the cytoplasm, since it results in a major loss of cations (Neupärtl et al. 2007). Because membrane voltage is the dominant driving force for uptake of many substances, such as sugars and amino acids, depolarization decreases these uptake processes (Agarkova et al. 2008). These changes certainly alter the physiological state of the infected cell. Second, cytoplasmic pH is altered early during infection (Agarkova et al. 2008). Cells generally control their cytoplasmic pH in a narrow range of ~7.5 (Felle 2005). Homeostasis of pH is essential because most enzymes in the cytoplasm are highly pH sensitive and operate over a narrow pH window. Any deviation in pH, therefore, has major consequences for the activity of cellular enzymes. PBCV-1 infection of *Chlorella* NC64A leads to alkalinization of the cytosolic pH (Fig. 1; Agarkova et al. 2008), a reaction, which downregulates the activity of most host enzymes. These are the only physiological effects we are currently aware of. However, one can predict that other cellular control factors might be altered during the early phase of infection, such as the concentration of cytosolic Ca^{2+}. This parameter is also controlled by membrane voltage and generally kept under strict homeostatic control; any deviation from a narrow set point results in a modification of numerous activities in cells (Sanders et al. 2002).

Collectively, these data and considerations lead to a plausible model on the physiological events associated with PBCV-1 infection. The virus induced depolarization and the subsequent modifications of the ionic strength, pH, and potentially Ca^{2+} are an efficient method for rapidly inhibiting the activity of cellular enzymes because they no longer operate at their optimum conditions. To understand the benefit for the virus, we only have to imagine that the viral proteins operate at a different pH optimum, ionic strength, etc., which resembles that created early during infection. This is a testable hypothesis for future experiments.

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The Role of Auxin in Root-Symbiont and Root-Pathogen Interactions: From Development to Defense

Ulrike Mathesius

Contents

Abstract Plants live in constant contact with microorganisms, many of which have profound effects on the growth and development of plant hosts. The plant hormone auxin regulates cell enlargement, cell division, and organogenesis, and is therefore a likely target for microorganisms that manipulate plants. In context of this chapter, the term microorganisms will be used to include bacteria, fungi, nematodes, and protozoans. Many microorganisms can synthesize auxin themselves. Others produce specific signals that indirectly alter the plant auxin balance, for example,

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through effects on auxin transport, metabolism, or signaling. This chapter highlights plant–microorganism interactions, in which auxin is targeted by symbionts and pathogens to manipulate the development of their plant host. Auxin signaling is also necessary for the regulation of plant defense responses against pathogens, and downregulation of auxin signaling has emerged as a strategy of plants to inhibit pathogen infection. Thus, the regulation of auxin signaling is a balancing act between influences of both the plant and the microbial partner. One future challenge will be to identify the microbial signal molecules that regulate the plant auxin balance, and to find out how the plant integrates the perception of several of these signals at the same time.

1 Introduction

Plants are colonized by a range of microorganisms that can live on the plant surface or inside plant tissues as endophytes. Many microorganisms alter plant architecture, for example, by enhancing or inhibiting plant growth or by inducing specific structures on the roots and stems that they colonize. For example, many plant-growth promoting rhizobacteria (PGPR) and mycorrhizal fungi stimulate root growth or root branching (Gianinazzi-Pearson 1996; Persello-Cartieaux et al. 2003); symbiotic nitrogen-fixing bacteria called rhizobia induce the formation of nodules on legume roots, which requires the reinitiation of the cell cycle in root pericycle and cortical cells (Foucher and Kondorosi 2000); plant parasitic nematodes cause the formation of root galls on a wide range of hosts, which involves extensive changes in cell division and differentiation (Goverse et al. 2000a); *Agrobacterium* species cause tumor formation in stems and roots of host plants (Escobar and Dandekar 2003), and pathogenic fungi and bacteria can lead to a range of root and shoot deformations through galling and fasciation (Jameson 2000).

In many cases, it has been suggested that these changes in plant development are mediated by the manipulation of the phytohormone balance by microorganisms (Hirsch et al. 1997). Several hormones have been associated with developmental changes caused by microbes in plants. Auxin is important in cell cycle regulation (Vanneste et al. 2005) and plant organogenesis (Kepinski and Leyser 2003) and therefore, likely to be involved in developmental changes caused by microorganisms. The correct localization, transport, and concentration of auxin is important in the formation of plant organs, and manipulation of auxin gradients leads to changes in plant growth and organ formation (Benkovà et al. 2003; Friml 2003). More recently, auxin has also been unmasked as a modulator of plant immunity (Lopez et al. 2008).

Microorganisms could alter plant development or defense by synthesizing auxin themselves, or by influencing auxin metabolism, signaling, or transport in the host. This chapter highlights some well-studied plant–microorganisms interactions and examines the possible mechanisms of auxin manipulation by microorganisms that are involved in developmental and defense responses in their hosts.

2 Regulation of Auxin Synthesis, Transport and Signaling in Plants

Auxin is synthesized by all higher plants. The most abundant form of auxin is indole-3-acetic acid (IAA), which can be synthesized from a tryptophan-dependent or a tryptophan-independent pathway (Woodward and Bartel 2005). A large pool of auxin is stored in a conjugated, inactive form, but can be reactivated through hydrolysis (Ljung et al. 2002). Oxidation can permanently inactivate auxin (Fig. 1). IAA is synthesized mainly in young shoot tissues and transported from there to other parts of the plant, although other tissues also have the capacity to synthesize auxin (Ljung et al. 2001; Ljung et al. 2005). Auxin is transported from the shoot to the root tip through the vascular tissue (Aloni 2004), and from the root tip to the elongation zone through epidermal cells (Mitchell and Davies 1975). Local transport of auxin along

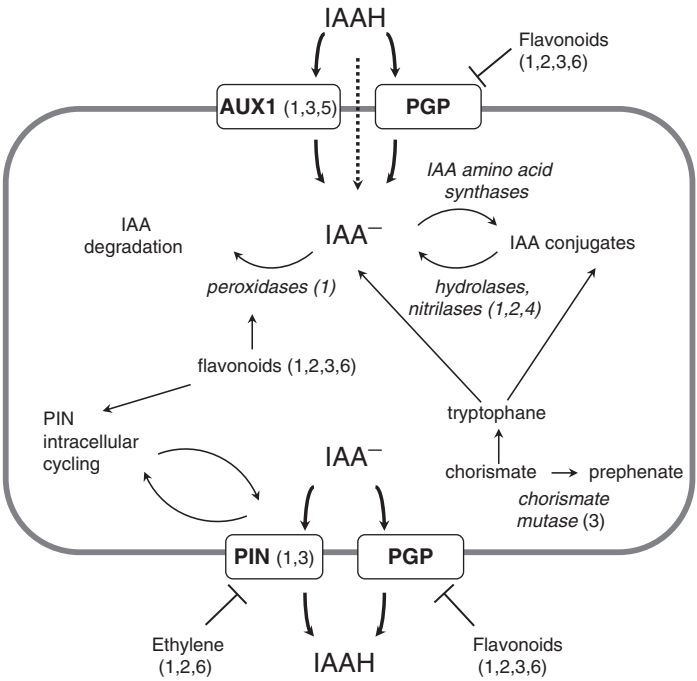

Fig. 1 Points of influence of microbial signals on auxin transport and metabolism. Auxin can diffuse into plant cells (*dashed arrow*) in the protonated form, but is additionally transported into cells by AUX1 and PGP transporters, and exported by PIN and PGP transporters, which can be regulated by flavonoids and ethylene. Auxin concentrations inside the cell are determined by auxin synthesis, degradation and conjugation. Microbes have been shown to directly or indirectly affect various regulators of auxin transport and metabolism as indicated by the following numbers: rhizobia (1), mycorrhizal fungi (2), plant parasitic nematodes (3), *Plasmodiophora brassicae* (4), *Frankia* sp. (5) and *Agrobacterium tumefaciens* (6). Image modified from Mathesius (2008)

and across tissues is important for auxin localization, for example, in emerging lateral root primordia (Jones 1998). At least two mechanisms of auxin transport have been demonstrated in plants, first via the phloem from source to sink tissues and second by active polar auxin transport (PAT) through auxin transport proteins (Fig. 1).

As a weak acid, auxin occurs in the protonated form (IAAH) in the acidic cell wall, and can diffuse inside cells. Auxin is also actively transported into cells by auxin importers of the amino acid permease families AUX1 (Auxin resistant 1), LAX [Like-AUX1] and PGP4, a member of the MDR/PGP (Multidrug resistance/P-glycoprotein) families (Terasaka et al. 2005; Yang et al. 2006). Because of the higher pH inside the cell, auxin deprotonates (IAA^-) and cannot diffuse out of the cell. Hence, auxin requires active export by transporters of the PIN (Pin-formed) and PGP families (Geisler et al. 2005; Petrasek et al. 2006). The polarity of auxin transport is determined by the polar localization of PIN proteins on either the basal or apical side of the cell (Wisniewska et al. 2006). Mutations or misexpression of *PIN* genes cause changes in auxin accumulation and organ development (Benkovà et al. 2003; Friml 2003; Vieten et al. 2007).

Auxin transport can be altered by regulating the activity, localization, and internalization of auxin transport proteins. The synthetic auxin transport inhibitors NPA (1-*N*-naphthylphalamic acid) and TIBA (2,3,5-triiodobenzoic acid) have been suggested to interfere with PIN activity by binding to MDR and PGP proteins, and by affecting intracellular cycling of PIN proteins between the plasma membrane and endosomal vesicles (Muday and DeLong 2001; Noh et al. 2001; Murphy et al. 2002; Geisler et al. 2005; Dhonukshe et al. 2008).

Certain flavonoids have been shown to act as natural auxin transport inhibitors through the regulation of PIN activity and localization (Fig. 1) (Peer and Murphy 2007). Flavonoids are phenylpropanoid metabolites of higher plants with a range of functions (Winkel-Shirley 2001). Specific flavonoids inhibit auxin transport by competing with synthetic auxin transport inhibitors (i.e. NPA and TIBA) for plasma membrane and microsomal binding sites (Stenlid 1976; Jacobs and Rubery 1988; Bernasconi 1996). Flavonoids can affect PAT by interacting with PGP proteins as well as with an aminopeptidase (Bernasconi 1996; Murphy and Taiz 1999). A lack of flavonoids in Arabidopsis mutants altered the expression and localization of certain PIN proteins, and it was suggested that flavonoids could act by targeting PIN intracellular cycling (Peer et al. 2004). Flavonoid-deficient plants show higher rates of auxin transport, whereas mutants over-accumulating flavonols show decreased auxin transport rates (Murphy et al. 2000; Brown et al. 2001; Peer et al. 2004; Wasson et al. 2006). However, PIN localization may not be directly regulated by flavonoids but by auxin localization itself in a positive feedback loop (Peer et al. 2004). Flavonoids could be ideal targets for the regulation of auxin transport by microbes because plants accumulate flavonoids in response to a variety of bacteria, fungi, and nematodes (Fig. 1) (Harrison and Dixon 1993; Dakora and Phillips 1996; Stafford 1997; Mathesius et al. 1998; Hutangura et al. 1999).

Ethylene can also inhibit auxin transport (Burg and Burg 1966; Morgan and Gausman 1966; Prayitno et al. 2006), although its mechanism of action remains

unclear. In *M. truncatula*, ethylene was shown to alter the expression of PIN proteins (Fig. 1) (Prayitno et al. 2006). Ethylene could also act via the induction of flavonoids in plants, which could then affect auxin transport (Buer et al. 2006). Similar to flavonoids, ethylene synthesis is affected by many plant-interacting bacteria and fungi (Guinel and Geil 2002; Gamalero et al. 2008) and could, thus, mediate changes in auxin transport or signaling.

Auxin has multiple effects in the plant, which are mediated through the action of auxin-regulated genes (Fig. 2). Auxin response genes contain promoter elements (Auxin-responsive elements, AuxRE) that are regulated by transcription factors of the ARF (Auxin Response Factor) family (Guilfoyle and Hagen 2001). In the absence of auxin, many auxin-regulated genes are repressed by a complex of ARF and Aux/IAA proteins binding to the AuxRE. Once auxin binds to one of its receptors TIR1 (Transport Inhibitor Response 1), AFB (Auxin Signaling F-Box) 1, 2, or 3 (Dharmasiri et al. 2005a, 2005b; Kepinski and Leyser 2005), AuxIAA proteins are degraded by ubiquitination. Degradation of Aux/IAA proteins occurs through the SCF^{TIR1} (SKP1, Cullin and F-box protein, in this case TIR1) complex, and leads to auxin-induced gene expression changes (Woodward and Bartel 2005; Badescu and Napier 2006; Tan et al. 2007).

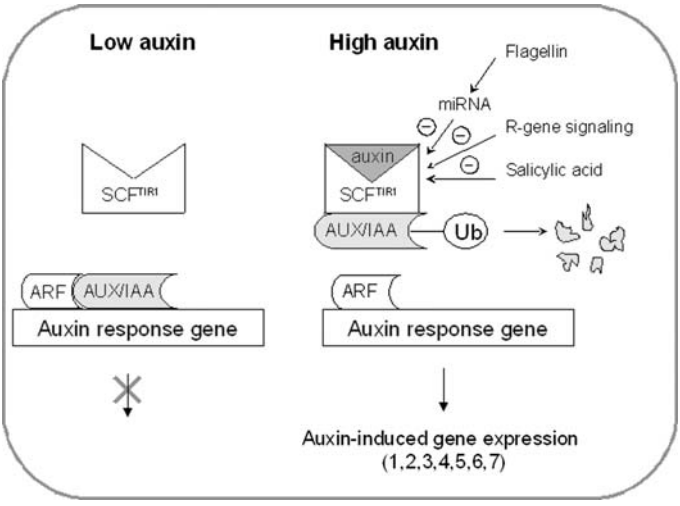

Fig. 2 Points of influence of microbial signals on auxin signaling. In the absence of auxin, auxin response genes are repressed by complexes of ARF and Aux/IAA proteins. Once auxin binds to its receptor, TIR1, an SCF^{TIR1} complex forms and leads to the breakdown of Aux/IAA through ubiquitination (Ub). This releases the repression of auxin response genes. Flagellin peptide, R gene signaling pathways and salicylic acid have been shown to inhibit auxin responses by down-regulating the auxin receptor TIR1. Microbes that have been shown to alter auxin induced genes expression are rhizobia (1), mycorrhizal fungi (2), plant parasitic nematodes (3), *Plasmodiophora brassicae* (4), *Frankia* sp. (5), and *Agrobacterium tumefaciens* (6). In addition, acyl homoserine lactone quorum sensing signals from gram-negative bacteria also affect auxin induced gene expression (7). Image modified from Mathesius (2008)

3 Microorganisms Synthesize Auxin to Alter Plant Growth

Many fungal and bacterial pathogens and symbionts directly synthesize auxin and thus affect auxin responses in the plant that are involved in symbiosis or pathogenicity. Since previous reviews have extensively covered the role of microbially synthesized auxin in plant–microbial interactions (Costacurta and Vanderleyden 1995; Spaepen et al. 2007), only a few examples will be highlighted here. Both pathogenic and symbiotic microorganisms synthesize auxin, and the exact role of auxin in these very diverse interactions has remained a question.

3.1 Auxin Synthesis by Tumor- and Gall-Forming Bacteria and Fungi

Several gall- and tumor-forming pathogens synthesize auxin or affect auxin synthesis or metabolism by the host. Auxin synthesis by *Pantoea* (*Erwinia*) *agglomerans* pv. *gypsophila* was shown to stimulate the formation of galls in its plant host *Gypsophila paniculata* (Clark et al. 1993; Barash and Manulis-Sasson 2007). Gall formation by *P. herbicola* pv. *gypsophilae* was significantly reduced in auxin synthesis mutants of the pathogen, suggesting that auxin plays an important role in pathogenesis (Manulis et al. 1998; Chalupowicz et al. 2006). *Agrobacterium tumefaciens*, *A. vitis,* and *A. rhizogenes*, soil-borne pathogens causing crown gall and hairy root disease, have been studied extensively for their ability to transform plant tissue. *A. tumefaciens* harbors auxin synthesis genes on its tumor-inducing (Ti) plasmid, which is transferred and integrated into the plant host DNA (Escobar and Dandekar 2003). Together with the synthesis of cytokinin, the synthesis of auxin causes massive proliferation of cells in stems and roots, leading to tumor formation. In addition to auxin synthesis, shoot-derived auxin is likely to be channeled into a growing tumor and retained there through the induction of flavonoids, which inhibit auxin export from the tumor (Schwalm et al. 2003). In addition, auxin-induced ethylene synthesis is likely to restrict auxin export from the tumor by causing narrowing of vessels (Aloni et al. 1995). This was confirmed by transferring an ACC deaminase gene (the product of which can degrade ACC, the immediate precursor of ethylene) from the plant growth promoting bacterium *Pseudomonas putida* into *A. tumefaciens*. This inhibited tumour development on both tomato and castor bean plants (Hao et al. 2007). Hairy roots are characterized by massive root proliferation, which in this case results primarily from the transfer of genes from the root-inducing (Ri) plasmid that alter auxin sensitivity, rather than auxin synthesis (Gelvin 1990).

The actinomycete *Rhodococcus fascians* can cause the formation of leafy galls, leaf malformations, and fasciation in a number of host plants (Goethals et al. 2001). Infected gall tissue is characterized by increased concentrations of auxin, although it is unclear whether the auxin source is the plant or the pathogen (Vereecke et al. 2000).

The smut fungus *Ustilago maydis* causes tumor formation on shoots and reproductive organs of a range of host plants. Tumors are characterized by increased auxin (IAA) concentrations. To investigate whether the auxin is synthesized by the fungal pathogen, Reineke and colleagues deleted several auxin synthesis genes from *U. maydis* (Reineke et al. 2008). Interestingly, while this led to decreased IAA concentrations in infected tissues, it did not prevent the development of tumors on host plants, suggesting that auxin synthesis, release of auxin from conjugates, or auxin signaling by the host could play a role in the formation of tumors.

While auxin appears to be an important hormone regulating tumor and gall formation by phytopathogens, its action is closely linked with the effects of cytokinin. Cytokinin synthesis is important in the cell proliferation of *Agrobacterium*-induced tumors (Veselov et al. 2003) and is also implicated in leafy gall formation by *R. fascians* (Depuydt et al. 2008). It has been shown that auxin can affect cytokinin synthesis and vice versa (Coenen and Lomax 1997; Miyawaki et al. 2004). Therefore, it would be difficult to isolate the effect of either hormones during the induction of cell proliferation by pathogens.

3.2 Auxin Synthesis in Plant Growth Promoting Rhizobacteria

PGPR have effects on plants, directly through production of enzymes and hormones, through nutrient cycling, and indirectly by inducing resistance mechanisms that protect plants from pathogens (Persello-Cartieaux et al. 2003). The synthesis of auxin and other hormones by rhizosphere bacteria is a common trait (Patten and Glick 1996). Auxin synthesis by PGPR can partially explain some of the growth-promoting effects that these bacteria have on plants, for example, the stimulation of root growth in wheat by *Azospirillum brasilense* (Dobbelaere et al. 1999), and in canola by *Pseudomonas putida* (Xie et al. 1996). In Arabidopsis, the effect of *P. thivervalensis* on root growth was shown to be attenuated in auxin-resistant mutants (Persello-Cartieaux et al. 2001). In contrast, *Bacillus megaterium*, which inhibits root elongation and promotes lateral root development, caused an increase in the expression of *DR5:GUS*, an auxin responsive promoter fusion, in lateral root primordia, but its effect on root growth and branching was not affected in a number of auxin and ethylene-insensitive Arabidopsis mutants (Lopez-Bucio et al. 2007).

3.3 Auxin Synthesis by Nitrogen-Fixing Endophytes

Cycads can form symbioses with nitrogen-fixing cyanobacteria, in which the symbiont induces the formation of so-called collaroid roots (Sprent and Sprent 1990). Auxin synthesis in cyanobacteria was found to be more common in symbiotic than in free-living species, and it was suggested that the auxin synthesized by these

cyanobacteria could activate mitotic divisions leading to collaroid root formation (Sergeeva et al. 2002). Actinomycetes can form symbioses with actinorhizal plants leading to the formation of lateral root-derived nodules, which are colonized by the bacteria (Pawlowski and Bisseling 1996). In the symbiosis between the actinomycete *Frankia* sp. and its host *Casuarina glauca,* the auxin import protein AUX1 was induced in root cells colonized by the symbiont (Peret et al. 2007). The authors suggested that auxin synthesized by *Frankia* sp. is transported into colonized host cells via AUX1 and that this is a necessary step in plant cell infection. Nitrogen-fixing rhizobia, which form symbioses with many legumes, also commonly synthesize auxin (Kefford et al. 1960). While the early steps of nodule initiation can be induced by Nod factors alone (see below), synthesis of IAA by rhizobia could be important at later stages of nodulation (Kefford et al. 1960). Studies with *Rhizobium* mutants deficient in IAA synthesis have shown that nitrogen fixation can be impaired by a lack of rhizobial auxin, whereas increased nodulation efficiency can be obtained with IAA overproducing strains (Pii et al. 2007).

4 Mechanisms of Auxin Regulation in Host Plants by Microorganisms

4.1 Signal Molecules of Bacteria with Effects on Host Auxin Signaling

While many plant-interacting bacteria synthesize auxin, it is also possible that their growth-promoting effects could be mediated by bacterial signaling molecules. One class of bacterial signals that has recently been shown to affect auxin signaling and root development are quorum sensing signals (QSS). QSS with species-specific structures are synthesized by most bacteria. Bacteria use QSS to communicate within a population and to coordinate cell density-dependent behaviors (Waters and Bassler 2005). QSS are especially important in plant–microbe interactions, and are crucial for plant-pathogenic (von Bodman et al. 2003) and symbiotic bacteria (Sanchez-Contreras et al. 2007), for example, for the regulation of biofilm formation, nitrogen fixation, synthesis of virulence factors, and bacterial motility. Not only do QSS serve as communication signals between bacteria, but also eukaryotic hosts have been shown to perceive QSS, which might enable them to preempt bacterial behaviors (Bauer and Mathesius 2004). Plants show extensive responses in their gene expression to QSS from gram-negative bacteria (acyl-homoserine lactones, AHLs), and these responses are specific to the structure of AHLs perceived (Mathesius et al. 2003). In *Medicago truncatula* (Mathesius et al. 2003) and *Arabidopsis thaliana* (von Rad et al. 2008), AHLs were shown to alter the expression of auxin-related genes and proteins. In *Trifolium repens*, this was accompanied by an induction of the auxin responsive reporter, *GH3:GUS*, in the roots (Mathesius et al. 2003). In Arabidopsis, AHLs also caused increased auxin concentrations (von Rad et al. 2008). Some of the AHLs were shown to have positive or negative

effects on root growth of Arabidopsis, including effects on the root apical meristem and lateral root density at micromolar concentrations (Ortiz-Castro et al. 2008; von Rad et al. 2008). However, the AHL C_{10}-homoserine lactone, which was shown to significantly affect root growth and development, was still active in three auxin response mutants (Ortiz-Castro et al. 2008). Therefore, even though AHLs appear to alter the plant auxin balance, it remains unclear to what extent the changes in auxin accumulation or signaling are responsible for developmental changes in plants. AHLs are synthesized by bacteria that can be beneficial, pathogenic, or neutral to plants, and while some of the AHLs synthesized by different bacteria can have the same chemical structure, the effects of the bacteria on host plants differ. It is possible that some of the effects of QSS on auxin could alter plant defense, rather than development (see Sect. 5).

Another class of bacterial signals that has been shown to have specific effects on root development through manipulation of plant auxin signaling are Nod factors synthesized by nitrogen-fixing bacteria called rhizobia. Their role is discussed in the next section.

4.2 Root Nodule Development by Rhizobia Involves Local and Systemic Changes in Auxin Transport

Nitrogen-fixing bacteria called rhizobia can form nodules on specific legume hosts. Exudation of flavonoids from host roots stimulates the synthesis of signal molecules called Nod factors (lipochitin oligosaccharides) by rhizobia, which are required for successful infection and nodule induction (Oldroyd and Downie 2008). Application of Nod factors alone can induce the early stages of nodule development in many legumes. Nodule organogenesis varies between different legume hosts. Nodules can either develop from modified lateral roots, or can initiate de novo in pericycle and cortical cells of host roots (Hirsch 1992). Indeterminate nodules are formed in many temperate legumes like pea and alfalfa; they originate from pericycle and inner cortical cell divisions and result in a nodule with a persistent meristem. Determinate nodules are typically formed on tropical legumes like beans and soybeans; they originate from outer cortical cell divisions and enlargements, which later fuse with a group of dividing pericycle cells. The stimulation of cell divisions in pericycle and cortical cells can be mimicked by alterations of the auxin and cytokinin ratio (Libbenga et al. 1973). Furthermore, the development of nodule-like structures on roots can be phenocopied by external application of synthetic auxin transport inhibitors to roots, suggesting that rhizobia induce nodules by interfering with PAT (Hirsch et al. 1989; Wu et al. 1996). This was confirmed by observations of reduced auxin response in clover roots expressing the *GH3:GUS* reporter at and below the site of inoculation of roots with rhizobia (Mathesius et al. 1998b). In addition, the transport of radiolabeled auxin was reduced in vetch roots inoculated with Nod factors (Boot et al. 1999), and silencing of *PIN* auxin transporters reduced nodulation in *M. truncatula* (Huo et al. 2006). Flavonoids were

hypothesized to mediate the inhibition of auxin transport by rhizobia, because flavonoids can act as natural auxin transport inhibitors (Jacobs and Rubery 1988), mimic the reduction of *GH3:GUS* expression seen in *Rhizobium*-inoculated roots (Mathesius et al. 1998b) and specifically accumulate at sites of *Rhizobium* infection (Mathesius et al. 1998a). Flavonoid-deficient roots of *M. truncatula* were subsequently shown to be defective in nodule development, and rhizobia were unable to inhibit auxin transport in these roots (Wasson et al. 2006). While these studies suggest that auxin transport inhibition is required for the initiation of indeterminate nodules, no auxin transport inhibition was detected during determinate nodule development (Pacios-Bras et al. 2003), and (iso)flavonoids were shown not to be required for nodule development in soybean (Subramanian et al. 2006). Therefore, flavonoids are likely to have different roles during determinate and indeterminate nodule development (Subramanian et al. 2007). Auxin transport inhibition appears to be specific for indeterminate nodulation, possibly reflecting the different requirements for cell division in either inner or outer cortical cells in the two types of nodules (Mathesius 2008).

The inhibition of auxin transport precedes the onset of cortical and pericycle cell divisions and might be required to determine the position of founder cells of nodule primordia (Mathesius 2008). Once founder cells are specified, auxin accumulates in dividing inner or outer cortical cells of both determinate (Pacios-Bras et al. 2003) and indeterminate (Mathesius et al. 1998b; van Noorden et al. 2007) nodule types, where auxin might be required for sustaining cell divisions (Foucher and Kondorosi 2000), similar to the accumulation of auxin in developing lateral root primordia (Benkovà et al. 2003). Auxin concentration changes are accompanied by changes in the activity of peroxidases, which can break down auxin (Fedorova et al. 2000), and peroxidase activity can be regulated by flavonoids, which accumulate in developing nodules (Mathesius 2001). In addition, rhizobia induce the expression of hydrolases that release auxin from conjugates (Campanella et al. 2008). Auxin is also likely to be transported into developing nodule primordia by auxin import via AUX1 and PIN transporters, which are strongly expressed in developing nodule primordia (de Billy et al. 2001; Huo et al. 2006). *PIN* expression can be regulated by ethylene during nodulation (Prayitno et al. 2006); whether *PINs* are also regulated by flavonoids during nodulation has not been established.

Not only do rhizobia affect the local auxin distribution and transport at the site of nodule development, but also alter shoot-to-root auxin transport via systemic signals. Inoculation of roots with rhizobia triggers "autoregulation," a systemic control that limits the numbers of nodules on a root system (Caetano-Anollés and Gresshoff 1991). Autoregulation mutants are defective in a leucine-rich receptor like kinase and supernodulate (Stacey et al. 2006). The *M. truncatula* autoregulation mutant *sunn* (*super numeric nodules*) is characterized by increased auxin transport from the shoot to the root (van Noorden et al. 2006). Whereas inoculation of wild type plants with rhizobia inhibits shoot-to-root auxin transport, auxin transport in *sunn* remains unaffected, suggesting that inhibition of systemic auxin transport is part of the autoregulation control (van Noorden et al. 2006). So far, none of the signals regulating the systemic control of auxin transport by rhizobia have been identified.

4.3 Mycorrhizal Fungi Affect the Root Auxin Balance

Symbiotic mycorrhizal fungi colonize the roots of a wide range of plants, and exchange phosphorus and other nutrients for host-derived carbon. Arbuscular mycorrhizal (AM) fungi colonize plants intra- and intercellularly and induce the formation of highly branched structures (arbuscules) inside root cortical cells, whereas ectomycorrhizal fungi produce extracellular mycelia that form a sheath around root tips and usually lead to root tip bifurcation and arrest of root growth (Gianinazzi-Pearson 1996; Barker et al. 1998; Harrison 2005). Both types of fungi can alter root development, usually by increasing the number of fine branch roots, and this could be a strategy to increase the number of infectable roots. As auxin is an important regulator of lateral root formation (Fukaki et al. 2007), it is possible that increased root branching is caused by enhanced auxin synthesis or signaling during symbiosis. Firstly, this could be a result of auxin synthesis by the fungal partner. Auxin is synthesized by both AM and ectomycorrhizal fungi and could have direct effects on hosts (Rudawska and Kieliszewska-Rokicka 1997; Sirrenberg et al. 2007). Secondly, mycorrhizal fungi have effects on the auxin synthesis of the infected host (Kaldorf and Ludwig-Müller 2000; Jentschel et al. 2007) and on the hydrolysis of auxin conjugates in the host (Fritze et al. 2005; Campanella et al. 2008). Root branching can be caused by a diffusible signal ("myc factor") exuded from the AM hyphae that has so far not been identified, but requires some of the same signal transduction genes necessary for the successful infection of legumes by rhizobia (Olah et al. 2005). The root branching could not be phenocopied by external application of auxin, suggesting that auxin is not, or not the only, the signal involved in root responses to AM fungi (Olah et al. 2005). However, a specific pattern of auxin localization inside roots might be required for developmental responses, and this is unlikely to be achieved by external auxin treatment. Localization of auxin responses using the *GH3:GUS* reporter showed uneven auxin accumulation in AM infected roots (Jentschel et al. 2007). Interestingly, mycorrhization can be stimulated by treatment of roots with Nod factors (which can inhibit auxin transport in legumes, see Sect. 4.2) and the synthetic auxin transport inhibitor TIBA, suggesting that changes in auxin transport might be part of AM formation (Xie et al. 1997).

4.4 The Role of Auxin in the Development of Clubroots by a Pathogenic Protist

The fungus-like protist *Plasmodiophora brassicae* causes clubroot disease in *Brassicaceae* by stimulating cell divisions and hypertrophy, which leads to deformed roots that become a strong nutrient sink and severely reduce plant growth (Ingram and Tommerup 1972). A significant increase in auxin content was measured in *P. brassicae*-infected roots (Ludwig-Müller et al. 1993; Devos et al. 2005) and different types of auxins and auxin conjugates, some derived from indole

glucosinolates, have been detected in clubroot tissue (Rausch et al. 1983). Increased expression of *DR5:GUS* was visible in infected Arabidopsis roots, and an Arabidopsis mutant defective in auxin–ethylene cross talk was less severely infected than control plants, suggesting that auxin is involved in clubroot formation (Devos et al. 2006). A model has been proposed in which *P. brassicae* alters the release of free auxin in plant tissues through effects on nitrilases and hydrolases that convert tryptophan and glucosinolates into free IAA (Fig. 1) (Rausch et al. 1983; Grsic-Rausch et al. 2000; Ludwig-Müller and Schuller 2008). While plant mutants defective in glucosinolate synthesis and auxin response did not show a straight correlation with resistance to the pathogen, it is likely that parallel pathways for auxin synthesis compensate for deficiencies in one branch (Ludwig-Müller et al. 1999; Ludwig-Müller and Schuller 2008). The signal molecules of *P. brassicae* that interfere with the host auxin metabolism are so far unknown. Whether *P. brassicae* itself synthesizes auxin has not been resolved, but it appears to be unlikely (Ludwig-Müller and Schuller 2008).

4.5 *Auxin is Involved in the Development of Root Galls by Parasitic Nematodes*

Plant parasitic nematodes induce the formation of root galls on many plant species. Cyst nematodes (*Globodera* ssp. and *Heterodera* ssp.) infect roots intracellularly and cause the development of cysts, which are typically initiated in pericycle, cortex, or procambium cells, and later incorporate xylem parenchyma cells that form a syncytium after cell wall dissolution and fusion of multiple cells (Goverse et al. 2000a). Root knot nematodes (*Meloidogyne* spp.) traverse the root tip intercellularly and stimulate parenchyma cells of the vascular bundle to endoreduplicate, resulting in the formation of multinucleate giant cells (Goverse et al. 2000a). In addition, extensive divisions of nearby pericycle and cortical cells often accompany the formation of these galls. In many cases, stimulation of pericycle cell division leads to additional development of lateral roots at the base of root galls (Goverse et al. 2000b; Karczmarek et al. 2004). Gall formation is initiated by secretions from the esophageal glands of nematodes, although it is not known which compound(s) contained in the secretions cause gall formation. Since the detection of auxin in root knot nematode galls (Balasubramanian and Rangaswami 1962; Yu and Viglierchio 1964), it has been suggested that auxin could play a role in the regulation of cell division and differentiation of root galls (Goverse et al. 2000b; Gheysen and Fenoll 2002). It is possible that some auxin is synthesized by the nematodes, although this has recently been questioned (Goverse et al. 2000b). Another possibility is that the infecting nematode secretions indirectly cause changes in auxin metabolism or transport by the host.

A number of genes encoding auxin response and auxin transport proteins were found to be upregulated in microarray experiments analyzing gene expression in

nematode galls (Gheysen and Fenoll 2002; Ithal et al. 2007a, 2007b; Klink et al. 2007), although some auxin response genes also showed downregulation (Ithal et al. 2007b). In addition, auxin-inducible cell cycle genes were upregulated (de Almeida-Engler et al. 1999) and auxin-repressed genes were downregulated in cyst nematode-infected roots (Hermsmeier et al. 1998). Increased expression of auxin responsive promoter:GUS fusions was demonstrated in early dividing cells, including feeding cells of galls induced by root knot (Hutangura et al. 1999; Karczmarek et al. 2004; Wang et al. 2007b) and cyst nematodes (Karczmarek et al. 2004; Wang et al. 2007b). At later stages of gall development, these auxin responses were downregulated. The requirement for auxin was confirmed by the fact that auxin-insensitive tomato and Arabidopsis mutants are defective in feeding cell establishment (Goverse et al. 2000). Interestingly, Arabidopsis mutants defective in auxin transporter-encoding genes were also impaired in syncytium development, and treatment of plants with the synthetic auxin transport inhibitor, NPA, caused severely distorted syncytium development and abnormal cell divisions, suggesting that nematodes could cause auxin accumulation by inhibiting auxin efflux (Goverse et al. 2000b). Similar to nodule development, this could be mediated by flavonoids, which accumulate in young galls (Hutangura et al. 1999). However, flavonoid-deficient mutants of Arabidopsis were still able to form feeding sites when infected with cyst nematodes (Jones et al. 2007). Alternatively, auxin could be channeled into forming galls and cysts through increased activity of auxin import proteins, and this is supported by strong expression of the gene encoding the auxin importer AUX1 in developing feeding structures of both cyst and root knot nematodes (Mazarei et al. 2003). Nematodes also secrete various enzymes into host tissue. Chorismate mutase is an enzyme injected into hosts that is involved in the synthesis of chorismate, a precursor for aromatic amino acids, phenylpropanoids, and the hormones, salicylic acid and auxin (Fig. 1). Overexpression of chorismate mutase in soybean roots caused abnormal lateral root development, and this phenotype was rescued by external auxin (Doyle and Lambert 2003). The authors suggested that cytoplasmic chorismate mutase could lower auxin concentrations in developing feeding structures by competing for chorismate substrates in the chloroplast, where auxin is synthesized, although this hypothesis is not supported by the previously mentioned demonstrations of increased auxin concentrations in developing feeding structures.

While the active compound(s) in nematode secretions that could be responsible for gall formation have not been identified, recent reports suggest that (some of) these active compounds might have been "hijacked" from rhizobia or plants. For example, NodL, an *N*-acetyltransferase, is likely to have been acquired by *Meloidogyne* sp. through horizontal gene transfer from rhizobia, where NodL is required for Nod factor synthesis (Scholl et al. 2003). Interestingly, secretions from nematodes also act through some of the same plant receptors required for Nod factor perception in legumes (Weerasinghe et al. 2005), and similar expression of meristem-specific genes is induced in developing galls and nodules, suggesting an overlap in signaling required for both organogenesis programs (Koltai et al. 2001). The presence of a peptide of the plant, CLE (CLAVATA3 /ENDOSPERM

SURROUNDING REGION) family of peptides, which regulate meristem differentiation in plants, was identified from *H. glycines* (Wang et al. 2005), suggesting that CLE peptides could be used by parasitic nematodes to regulate meristematic activity required for gall formation through ligand mimicry (Mitchum et al. 2008). It would be interesting to test whether nematode-derived CLE peptides affect gall formation by interfering with auxin signaling, because of the critical role of auxin in meristem activity (Friml 2003).

5 A Role for Auxin in Plant Defense Against Pathogens

Apart from the role of auxin as a developmental regulator of root–microbe interactions, a growing number of studies have shown that auxin mediates changes in plant defense responses during infection with pathogenic bacteria and fungi. Two mechanisms of resistance to pathogens are known to occur in plants (Jones and Dangl 2006). First, plants have a basal defense system triggered upon recognition of conserved microbial molecules or "pathogen-associated molecular patterns" (PAMPs). This defense system is active in host and nonhost species and acts as a first barrier to infection. Second, in many specific plant–pathogen interactions, disease suppression is mediated by resistance (*R*) genes of the plant host. The encoded proteins of *R* genes specifically interact with pathogen avirulence proteins, leading to hypersensitive responses. Both defense systems involve activation of auxin signaling. There is evidence that pathogens affect auxin signaling as part of their infection process, and that downregulation of auxin signaling by the plant is a strategy to enhance pathogen resistance (Lopez et al. 2008; Spoel and Dong 2008).

5.1 Pathogens Affect Auxin Signaling

There is evidence that pathogens can enhance auxin synthesis and/or signaling in host plants, and that this is required for infection. For example, the plant pathogen *Pseudomonas syringae* was shown to have extensive effects on auxin-regulated gene expression in Arabidopsis, including changes to auxin synthesis and auxin transport genes (Thilmony et al. 2006). The *P. synringae* type III effector, AvrRpt2, increased auxin sensitivity and auxin concentrations in Arabidopsis, and this was correlated with increased pathogen virulence (Chen et al. 2007). In Arabidopsis, the defense related gene *CEVI-1*, which was initially cloned from tomato and is induced upon infection by viral pathogens, was shown to be under negative control of genes regulating auxin sensitivity (Mayda et al. 2000). External auxin application can enhance pathogenicity symptoms, for example, those caused by *Pythium ultimatum* on tomato plants (Gravel et al. 2007). Coinoculation of Arabidopsis plants with auxin and the pathogen *P. syringae*

caused enhanced disease symptoms, whereas an auxin-insensitive mutant, *axr2-2*, was more resistant to *P. syringae* (Wang et al. 2007a). Similarly, an Arabidopsis auxin-insensitive mutant was found to be resistant to infection by *P. thirvervalensis* (Persello-Cartieaux et al. 2001).

Two *R*-gene products from Arabidopsis, SGT1b (Austin et al. 2002) and its interactor RAR1 (Azevedo et al. 2002) were found to be required for resistance to downy mildew. The SGT1b protein was subsequently characterized in a screen for factors required for activation of the SCF^{TIR1} complex (Gray et al. 2003), which is involved in the proteolytic degradation of auxin-regulated proteins through ubiquitination (Fig. 2) (Gray et al. 2001). These studies suggest that auxin signaling is required for (certain) *R*-gene mediated resistance mechanisms. This was confirmed by a microarray experiment comparing auxin responses in wild type and auxin resistant Arabidopsis plants, which identified several disease-related proteins, including *R* gene family members, as auxin-regulated (Huang et al. 2008). A proteome study evaluating protein responses in *M. truncatula* to auxin also found multiple defense-related genes as targets of auxin (van Noorden et al. 2007).

5.2 The Plant Downregulates Auxin Signaling as Part of a Defense Response

A common PAMP triggering basal defense response in plants is the flagellin peptide, derived from bacterial flagellin. Exposure of Arabidopsis to a flagellin-derived peptide induced the production of a microRNA, which was shown to decrease the expression of *TIR1*, encoding an auxin receptor (Navarro et al. 2006). The subsequently reduced auxin response in the plants was linked to (nonrace-specific) resistance of Arabidopsis to pathogens (Navarro et al. 2006). Salicylic acid (SA) mediates disease resistance mechanisms against a range of pathogens. SA was found to inhibit the auxin receptor TIR1, causing stabilization of AUX/IAA proteins and a subsequently reduced auxin response (Wang et al. 2007). Thus, the auxin receptor TIR1 appears to be an important target of plant-mediated downregulation of auxin signaling as part of a defense strategy (Fig. 2).

The auxin response gene *GH3.5*, an IAA amino acid synthase controlling auxin conjugation to amino acids, was shown to regulate both auxin and SA-mediated responses to *P. syringae* in Arabidopsis (Zhang et al. 2007). The authors suggested that GH3.5 mediates a reduction of auxin synthesis in the host as part of the resistance mechanism. Similarly, a related early auxin response gene, *GH3-8*, was reported to activate disease resistance in rice against *Xanthomonas oryzae* pv *oryzae* (Ding et al. 2008). Over-expression of *GH3-8* caused enhanced disease resistance, and this was accompanied by reduced free auxin accumulation and suppressed auxin signaling.

6 Conclusions and Perspectives

Microorganisms have developed a multitude of strategies to alter plant development and defense using auxin. These strategies include the synthesis of auxin by the microbes (many PGPRs), integration of auxin synthesis genes into the plant genome (*Agrobacterium* ssp.), manipulation of auxin accumulation and metabolism (rhizobia, *P. brassicae* and plant parasitic nematodes), possibly through alteration of auxin import and export or via changes to auxin degradation (Table 1). For most plant–microbial interactions, neither the detailed mechanism of how microbes alter auxin accumulation nor the active microbial signals are known. For the *Rhizobium*–legume and AM symbioses, which are not formed in Arabidopsis, the main limitation has been the lack of auxin-related mutants, but advances have been made with selective gene silencing using RNA interference. Furthermore, the identification of microbial signal molecules, in particular those of mycorrhizal fungi and plant parasitic nematodes, and their receptors in plants would be an important advance in trying to understand the mechanism of action of these signals on plant development.

Another area of future research that has so far largely been neglected is how the soil ecology feeds back on plant architecture. For example, a further mechanism by which plant architecture could be modified by microorganisms was shown to operate by selective grazing of soil protozoa on rhizosphere bacteria. Protozoa are known to stimulate lateral root formation in many plants, and it has been suggested that this could be due to either nutrient or hormonal effects on plant roots, including direct contributions from protozoa or indirect contributions from soil bacteria that protozoa graze on (Bonkowski 2004). Interestingly, certain protozoa cause a relative increase in the percentage of IAA-producing rhizobacteria in the rhizosphere of watercress seedlings, and this could explain the enhanced root branching (Bonkowski and Brandt 2002).

While auxin signaling is often enhanced by plant pathogens, hosts have evolved counter-strategies to downregulate auxin signaling as part of a defense response. An important question for the future will be to determine whether the changes in auxin accumulation induced by symbionts will also lead to altered defense responses, or whether only specific signals from pathogens induce auxin changes in the plant that result in defense responses by the plant. Another unstudied question remains how the plant integrates multiple inputs from different microorganisms at the same time. Most studies are done with one microbial species, whereas plants living in soil are exposed to many species of microorganisms at the same time. For example, it would be interesting to test the simultaneous effect of symbionts and pathogens, or their specific signal molecules, on the plant auxin balance and resulting defense responses. The knowledge gained in these studies might enable us to use soil microorganisms in a more targeted way to improve the growth and architecture of plants.

In addition to studying the role of auxin in the developmental regulation by microbes, other phytohormones play important roles. For example, changes in

Table 1 Mechanisms of auxin regulation in plant hosts by various microbes, fungi and nematodes

Organism	Signal molecules	Effects on host auxin content, transport or signaling	Suggested mechanisms
Bacteria			
Rhizobia (*Sinorhizobium meliloti, Rhizobium leguminosarum*)	Nod factors	Local auxin transport inhibition, followed by increased auxin accumulation during nodule initiation	Induction of flavonoids that inhibit polar auxin transport; Induction of ethylene (signaling) that interferes with auxin transport via *PIN* gene expression
		Long distance auxin transport inhibition as part of autoregulation	Unknown mechanism regulated by autoregulation gene
Agrobacterium tumefaciens	Ti plasmid	Increased auxin synthesis in transformed tumors	Transfer of auxin synthesis genes into host DNA
Agrobacterium rhizogenes	Ri plasmid	Increased auxin sensitivity in transformed hairy roots	Transfer of genes that alter auxin sensitivity into host DNA
Pseudomonas syringae	Type III effector AvrRpt2	Increased auxin sensitivity and content	Unknown
Various gram-negative bacteria	Acyl homoserine lactone quorum sensing signals	Regulation of auxin response genes, increased auxin content	Unknown
Various species	Flagellin peptide	Decreased auxin signaling	Induction of miRNA that inhibits TIR1 auxin receptor
Fungi			
AM fungi	"Myc factor" (unidentified)	Increased auxin synthesis and response Altered auxin metabolism	Unknown; possible involvement of auxin transport machinery
Fungi-like protista			
Plasmodiophora brassicae	Unknown	Increased auxin synthesis and response Altered auxin metabolism	Activation of enzymes (nitrilases, hydrolases) in host tissue that hydrolyse auxin conjugates
Nematodes			
Cyst nematodes	Unknown pharyngeal gland secretions	Enhanced auxin response at early stages of infection Altered auxin transporter expression	Unknown
Root knot nematodes	Unknown pharyngeal gland secretions	Enhanced auxin response at early stages of infection Altered auxin transporter expression	Possible alteration of auxin metabolism by injection of chorismate mutase, and other unknown mechanisms

cytokinin metabolism and signaling are important in nodulation (Lohar et al. 2004; Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Tirichine et al. 2007), in AM symbioses (Hirsch and Kapulnik 1998; Barker and Tagu 2000), and nematode gall formation (Lohar et al. 2004). It will be important to understand the interactions of auxin with other hormones to build a more complete model of how auxin mediates microbial influences on plants.

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Molecular Mechanism of Water and Gas Transport Across Biological Membranes and Consequences for Plant Physiology

Norbert Uehlein

Contents

Abstract Aquaporins are membrane proteins, facilitating the transport of water across biological membranes. At the time of their discovery, biological membranes were thought to be that permeable for water, that there was no need for proteins facilitating membrane water transport. In fact, the demonstration of aquaporin function was so groundbreaking, that in 2003 the Nobel Prize for Chemistry was awarded to Peter Agre for his discovery of the aquaporins. Another property of

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certain aquaporins identified in recent years relates to facilitating membrane transport of gasses like CO_2 or NH_3. This function was also widely thought to be unnecessary, as membranes were believed to be permeable to gasses in general.

In plants, there are many processes where regulation of aquaporin expression and activity is very important. This chapter mainly focuses on involvement of plasma membrane intrinsic aquaporins. PIP1 and PIP2 isoforms are expressed in all parts or organs of the plant. All these differ in morphology and function, and therefore, in their requirement to membrane transport of water or CO_2. A striking relevance of aquaporin expression and activity could not only be shown for processes, which are obviously depending on water or CO_2, like root water uptake or photosynthesis, but also for plant reproduction, leaf movements, symbiosis, and other processes. Analyzing the role of aquaporins throughout the plant it appears, that in different organs the same type of aquaporin can perform different tasks. It seems that function of aquaporins can be modified according to the requirements of the tissue or organ where they are expressed. In the past, many different approaches to characterize aquaporins were adopted, highlighting a particular contribution of aquaporins on the level of molecules, cells, tissues or complete organisms, also including theoretical and computational analyses and modeling. The term "systems biology" was established to describe interdisciplinary studies on complex interactions in biological systems. The total work done in the field of aquaporins to date is a solid example of systems biology.

1 Membrane Transport of Water and Gasses

1.1 Membranes as Resistances to Substance Transport and Diffusion

Biological membranes are amphiphilic layers that surround cells or enclose subcellular compartments. Basically, they consist of a phospholipid bilayer with integral or peripheral protein components inserted into the lipid matrix. In general, membranes define compartments in which distinct chemical conditions are maintained, differing from those on the outside. In order to support chemical gradients, membranes have to impose resistances towards uncontrolled substance transport or diffusion and have to control which amount of a substance crosses the membrane.

Selective membrane permeability is fundamental to all processes of life. Membrane transport of ions, nutrients, solutes, and excretory substances depends upon a number of complex transport mechanisms. All together, controlled import into and export from a cell or compartment is the basis for proper functioning of an organism. Size, charge and hydropathy of a molecule determine if it can cross a membrane or not.

Already in 1899, Ernest Overton, one of the pioneers in lipid membrane research introduced a wide-spread theory of lipid membrane structure and function: The permeability of a molecule across a lipid membrane is related to the molecules

size and its solubility in lipid. From today's point of view, Overton's rule is no longer generally acceptable (Al-Awqati 1999), but it basically means substances are more likely to pass a membrane if they are hydrophobic. However, many key molecules to be transported into and out of a cell are hydrophilic like ions, sugars, and of course water, and hence are not covered by Overton's rule. In order to allow proper substance exchange, membranes contain numerous proteinacious transport systems like ion channels, amino acid transporters, proton pumps, and many more.

Key molecules that have to be exchanged by all living organisms are water and gasses like CO_2 and O_2. The generally accepted concept is that water and gasses can freely permeate cellular membranes by simple diffusion and there is definitely no need for proteinacious transport systems. Free diffusion of water across a lipid bilayer or a biomembrane would clearly contradict Overton's rule (Cooper et al. 2002; Hevesy et al. 1935).

1.2 The Diffusion Dogma – Part I: Membrane Water Transport

The water permeability of cellular membranes can differ considerably. Some 70 years ago, Hevesy and colleagues reported unexpectedly high transepithelial water flow across amphibian skin, which is about five-times higher than calculated for simple diffusion across a lipid bilayer (Hevesy et al. 1935). The red blood cell membrane was found to exhibit a similarly high water permeability, allowing rapid swelling or shrinking of the erythrocytes in response to small changes in osmolality (Macey 1984; Solomon et al. 1983). Effective reabsorption of water from primary urine in mammalian renal epithelia requires a high water permeability of the responsible cells (Verkman 1989). In contrast to these membranes displaying unusual high water permeability, other membranes, like the plasma membrane of rat urinary bladder endothelia were shown to exhibit very low water permeability (Hicks et al. 1974; Hicks and Ketterer 1970). These findings contradict the common opinion that membranes are highly permeable for water in general. The wide range of membrane water permeability suggested a proteinacious component facilitating or regulating water transport and determining the water permeability of membranes.

This proposed proteinacious water transport system was first identified with a channel forming integral membrane protein of 28 kDa (CHIP28) that was found to function as a water conducting pore in erythrocytes, kidney proximal tubule, and thin descending limb of loop of Henle (Denker et al. 1988; Preston et al. 1992; Smith and Agre 1991; Verbavatz et al. 1993). Based on its function as a water channel in humans, CHIP28 was later named *Homo sapiens* aquaporin 1 (HsAQP1), which has also been shown to be the site of the Colton blood group antigen (Smith et al. 1994). Meanwhile, aquaporins were identified in most kinds of living organisms. Already, very early aquaporin studies showed that there are aquaporin proteins, which are not strictly water selective, but can also conduct small neutral solutes. These are, for example, glycerol, urea, formamide, and hydrogen peroxide (Biela et al. 1999; Gerbeau et al. 1999; Kaldenhoff and Fischer 2006b).

Based on functional properties and sequence similarities, plant aquaporins are divided into four distinct subfamilies: PIP (plasma membrane intrinsic proteins), TIP (tonoplast intrinsic proteins), NIP (nodulin 26-like intrinsic proteins), and SIP (small and basic intrinsic proteins). The first aquaporin encoding sequences identified from plants, later turned out to be members of the NIP subfamily (Sandal and Marcker 1988). They were named after the aquaporin Nod26, which was initially found in the peribacteroid membrane of nodulated soybean roots (Niemietz and Tyerman 2000). Some NIPs have glycerol transport activity (Dean et al. 1999) and thus, can be regarded as plant glycerol transporters. Members of this subfamily were also found in nonlegume plants.

PIP aquaporins were subdivided into two discrete subgroups referred to as PIP1 and PIP2, respectively. Analyses in heterologous expression systems revealed tremendous functional differences between the subgroups. It was observed that expression of PIP1 aquaporins does not increase the membrane water permeability considerably (Biela et al. 1999), whereas it increases upon expression of PIP2 aquaporins by 20- to 50-fold (Fetter et al. 2004; Moshelion et al. 2002). The same functional difference is also commonly observed in the yeast expression system. The molecular reason of this difference in water conductivity is not yet clarified, however, analyses in the past years have shown that it must be due to protein properties or regulatory mechanisms, rather than integration of different protein amounts into membranes.

1.3 The Diffusion Dogma – Part II: Membrane Gas Transport

Aquaporins were initially characterized as membrane water transport systems. However, in the past few years, hints emerged that besides water, aquaporins can also facilitate membrane flux of gasses like carbon dioxide and ammonia. The common notion was, and to some extent, still is that there is no need for a proteinacious transport system as biomembranes do not build up resistances for gas transport and gasses simply diffuse across the lipid bilayer. Indeed the gas permeability of lipid bilayers is very high. As it was shown already in 1977 by Gutknecht and coworkers, the CO_2 permeability coefficient (P_{CO2}) of an artificial lecithin/cholesterol membrane is 35 cm s^{-1} (Gutknecht et al. 1977). However, in living organisms, there are no simple lipid bilayers, but biomembranes. Biomembranes contain regions varying in thickness and lipid composition and are associated with integral and peripheral protein components, limiting the exposure of free membrane area to adjacent aqueous compartments (Engelman 2005).

In living organisms, gas permeability of membranes can vary considerably – even within individual cells. Fermentation of nonabsorbed nutrients in the colon generates high concentrations of NH_3/NH_4^+ in the colonic lumen. NH_3 is a small, lipophilic, neutral, weak base that can permeate almost all cell membranes and affect the pH of the cytoplasm. However, colonocytes are able to maintain intracellular pH in the unusual acid–base environment of the colon. In isolated colonic

crypts, it has been observed that cytosolic pH of the epithelial cells does not increase when the NH_3 concentration in the colon lumen is raised, but does so immediately when it is increased on the basolateral side (Singh et al. 1995).

The CO_2 permeability of apical membranes of gastro-intestinal endothelia is very low (Endeward and Gros 2005), obviously contradicting the common view of gas permeable membranes. Consequently, it was concluded that there must be a permeability barrier to gasses in certain types of membranes. Some bacteria generate very high CO_2 partial pressures of up to 50.66 kPa (Rasmussen et al. 1999, 2002) in the colon lumen. Extreme CO_2 partial pressure can seriously affect the pH of epithelial cells, if it can freely permeate across the plasma membrane into the cytosol. Thus, it appears to be quite reasonable from a physiological point of view, if some membranes build up a gas barrier to protect the cells. By comparison in organs and tissues important for gas exchange, membrane gas permeability, e.g., in lung alveolar endothelia and red blood cells (Endeward et al. 2006b), is very high, to assure a quick removal of gaseous waste products.

It has been shown that aquaporins can facilitate membrane transport of CO_2. First evidence came from research on the human aquaporin HsAQP1. *Xenopus* oocytes are a useful tool to analyze functional properties of aquaporins, as they generally exhibit a low water and also a low CO_2 permeability of the plasma membrane. Using these as an expression system, it was shown that HsAQP1 considerably increases their membrane CO_2 permeability (Nakhoul et al. 1998). Further evidence came from analyses on human red blood cells (Endeward et al. 2006a, 2006b) that express human AQP1. However, a clear physiological relevance could not be shown (Fang et al. 2002).

Further support for the concept of aquaporin facilitated membrane transport of gasses comes from theoretical and computational biophysics. Molecular dynamics simulations showed that CO_2 or NH_3 molecules are small enough to pass through aquaporin pores and, therefore, aquaporin mediated transport is possible in principle (Hub and de Groot 2006; Hub and de Groot 2008), especially, if the intrinsic CO_2 permeability of the membrane is low.

2 Aquaporins in General

2.1 Aquaporin Structure

All aquaporin proteins exhibit a characteristic conserved structure. Hydropathy plot analyses of the primary sequence predicted a topology of six transmembrane helices (I–VI) connected by five loops (loops A–E; Fig. 1).

Loops A, C, and E are extracellular and loops B and D are intracellular. The protein comprises two internal tandem repeats, covering roughly the amino- and carboxy-terminal halves of the protein. Each repeat consists of three transmembrane helices and a highly conserved loop following the second transmembrane helix (loops B and E, respectively). These loops include a conserved signature motif,

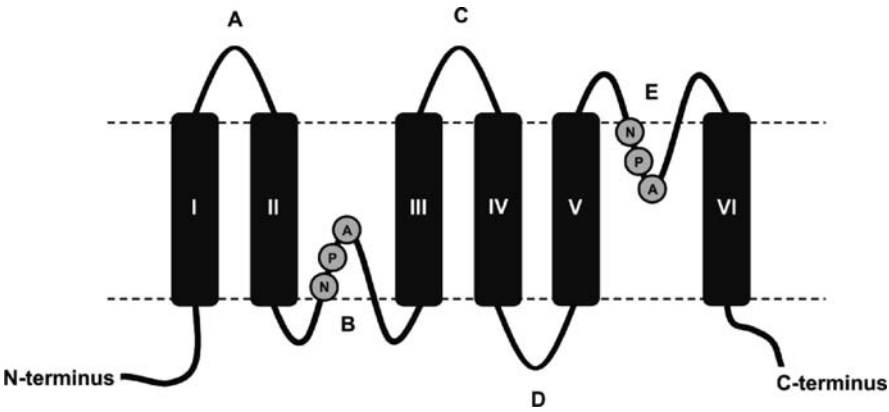

Fig. 1 General topology outline of aquaporins comprising six transmembrane helices connected by five loops (A–E), and comprising two functionally essential amino acid motives asparagine–proline–alanine (NPA)

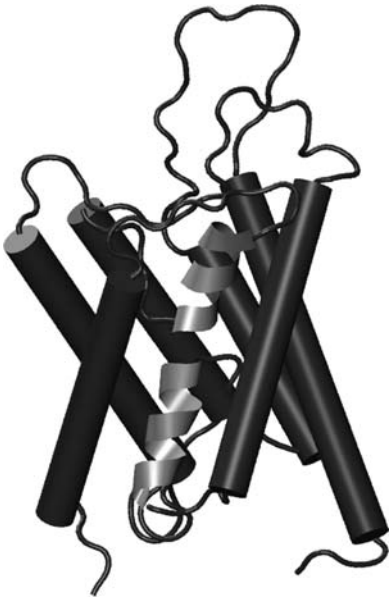

Fig. 2 Homology model of the PIP1 aquaporin NtAQP1 from *Nicotiana tabacum* (Biela et al. 1999; Siefritz et al. 2002; Uehlein et al. 2003; Uehlein et al. 2008). Loops B and E carrying the functionally important NPA boxes are shown in *light-gray*. The model was produced using MODELLER (http://www.salilab.org/modeller/) and Visual Molecular Dynamics (http://www.ks.uiuc.edu/Research/vmd/

asparagine–proline–alanine (NPA). Loops B and E form short α-helices that fold back into the membrane from opposite sides. As a consequence, a seventh transmembrane domain in which the two NPA boxes meet in the center of the membrane is formed (Fig. 2). This arrangement of helices was further referred to as the "hourglass model" (Jung et al. 1994). Molecular dynamics simulations showed that the NPA motives and another constriction site, the ar/R-region, are important for channel-selectivity and for guiding water molecules in a single file arrangement

through the pore (de Groot et al. 2000; de Groot and Grubmuller 2001; Jung et al. 1994; Murata et al. 2000). An excellent collection of illustrations and information for further reading in this context is available at http://www.mpibpc.mpg.de/groups/de_groot/gallery.html and http://www.ks.uiuc.edu/Research/aquaporins/.

This "hourglass model" has been confirmed by crystallographic studies on human AQP1 using cryo-electron microscopy and X-ray crystallography (de Groot et al. 2000; de Groot et al. 2003). The studies also showed that aquaporins have a tetrameric arrangement, in which four subunits line a putative fifth pore in the center of the tetramer. It is generally accepted that all aquaporin-like proteins assemble into tetramers, however, not necessarily important for function. Each monomer alone can facilitate water flow. Further studies have indicated conductance of ions (K+, Cs+, Na+ and tetramethylammonium) through the central pore of the AQP1 tetramer (Saparov et al. 2001; Yool and Weinstein 2002).

2.2 *Plant Aquaporins*

The basic structure of aquaporins is similar in all kingdoms of live, but the number of aquaporin genes is strikingly diverse in different organisms. Currently, 13 aquaporins are known in humans (Magni et al. 2006), which are divided into two subgroups according to their substrate specificity, whereas the model plant *Arabidopsis thaliana* has 35 aquaporin homologs (Johanson et al. 2001; Quigley et al. 2001). An equally high number of 36 aquaporin genes was found in maize (Chaumont et al. 2001) and 33 aquaporin genes could be detected in rice (Sakurai et al. 2005). The exceedingly high abundance of aquaporin genes in plants very likely results from the higher degree of compartmentation of plant cells versus animal cells. In addition, plants, as they are usually sessile, are in a greater need for fine tuned water control to adapt to regularly changing or to cope with hostile environmental conditions (Kaldenhoff and Fischer 2006a; Kaldenhoff and Fischer 2006b; Kruse et al. 2006).

Plant aquaporins are located in the plasma membrane and in intracellular membranes of plant cells, where they facilitate the transport of water and small neutral solutes like urea, boric acid, silicic acid, glycine, H_2O_2, and formamide or gasses like ammonia and carbon dioxide (Kaldenhoff and Fischer 2006b). However, only for water, ammonia and carbon dioxide a physiological relevance could be shown.

2.3 *Regulation of Aquaporin Activity*

It is quite obvious that a rapid and fine tuned regulation of membrane water transport is particularly important for plants as they cannot escape harmful conditions like drought- or salt-stress, or flooding. This could explain the exceptionally high number of aquaporin encoding genes in plants. Plants have to adjust their membrane permeability to water in response to different environmental challenges.

A couple of regulatory mechanisms are subsequently discussed, i.e., heteromerization of different aquaporin isoforms and molecular trafficking, aquaporin gating, and posttranslational modification.

2.3.1 Heteromultimerization and Membrane Targeting

It has been shown in the past that multimerization of membrane proteins can regulate their activity and function, as it was, for example, shown for sugar transporters and ion channels (Dreyer et al. 1997; Veenhoff et al. 2001). The effect of heteromerization on water and CO_2 conductivity, respectively, was tested on artificially generated heterotetramers using a heterologous expression system (Fischer 2007; Pede 2008). Fusion proteins containing different ratios of NtAQP1 (Biela et al. 1999) and NtPIP2;1 (Bots et al. 2005a) were expressed in *Saccharomyces cerevisiae*. Appearance of NtAQP1 monomers in the tetramer favored CO_2 permeability and reduced water permeability of the yeast plasma membrane, whereas the contrary effect could be observed when more NtPIP2;1 units were present in the tetramer (Fischer and Kaldenhoff, unpublished data).

The extraordinary abundance of aquaporins in plants is not yet fully understood. Most remarkable and puzzling is that PIP1 subfamily members, which are generally expressed at high levels (Otto and Kaldenhoff, unpublished data), hardly show water transport function in any testing system. Chaumont and coworkers offer a possible explanation for that phenomenon. Initially, they showed that in *Xenopus* oocytes PIP1;2 and PIP2;1 from *Zea mays* interact to increase the water permeability of the plasma membrane (Fetter et al. 2004). Subsequently, they transiently expressed fusions with fluorescent proteins in living maize cells. When expressed alone, ZmPIP1 fusion proteins remained in the endoplasmic reticulum, whereas ZmPIP2s were found in the plasma membrane. However, as analyzed by the FRET technique, upon coexpression, both aquaporins interacted and were targeted to the plasma membrane. Interestingly, when coexpressed with ZmPIP2s, ZmPIP1s were relocalized to the plasma membrane. These data suggest that PIP1–PIP2 interaction is required for PIP1 trafficking to the plasma membrane *in planta* to modulate plasma membrane permeability (Zelazny et al. 2007). However, upon expression of the aquaporin PIP1;2 from tobacco (NtAQP1) in heterologous systems without coexpression of a PIP2 member, it can be detected in the plasma membrane by western blot as well as by functional tests (Uehlein et al. 2003; Fischer 2007). Thus, it can be concluded that interaction of PIP1 and PIP2 has an effect on PIP1 trafficking, but also a certain amount of PIP1 alone finds its way to the plasma membrane.

2.3.2 Aquaporin Gating

Recently, a model of aquaporin gating has been proposed (Törnroth-Horsefield et al. 2006). The authors analyzed the crystal structures of the spinach plasma membrane aquaporin SoPIP2;1 crystallized in a closed and an open conformation. In the closed conformation, loop D (see Fig. 1) blocks the channel from the

cytoplasmic side by covering the pore, whereas it is displaced from the pore in the open conformation. Channel closure in that specific case is achieved by two different mechanisms: by dephosphorylation of two conserved serine residues under drought stress conditions, or by protonation of a conserved histidine residue in response to a drop in cytoplasmic pH following anoxia during flooding. Due to the high sequence conservation at the relevant sites, this model was suggested to be universal to all plant PIPs.

"However, a functional analysis of tobacco NtAQP1 and NtPIP2;1 and respective mutants did not support the gating mechanism prediction as a consequence of the combined effects from phosphorylation and protonation (Fischer and Kaldenhoff 2008)."

2.3.3 Post-Translational Modification

Using a mass spectrometry approach, Santoni et al. (2006) observed that the N-terminal part of PIP aquaporins can exhibit multiple modifications and is differentially processed between members of the PIP1 and PIP2 subclasses. They showed that the initiating methionine was acetylated or cleaved in native PIP1 and PIP2 aquaporins. More interesting, several residues are methylated in PIP2 aquaporins and could potentially account for the differences in water conductivity between PIP1 and PIP2 aquaporins. To analyze that, wild type AthPIP2;1 and mutant forms with altered methylation sites were expressed in *Arabidopsis* suspension cells. However, water transport measurements on vesicles purified from these cells suggested that PIP2;1 methylation does not interfere with the aquaporin intrinsic water permeability.

Methylation was identified as a novel posttranslational modification of aquaporins and maybe also other plant membrane proteins that does not interfere with functional characteristics of aquaporins, but may play a role in protein stability and subcellular localization (Santoni et al. 2006).

3 Summary of Aquaporin Dependent Processes in Plants

In plants, there are many processes where regulation of aquaporin expression and activity is very important for physiology. The following collection of work will mainly focus on involvement of plasma membrane intrinsic aquaporins. PIP1 and PIP2 isoforms are expressed in nearly all parts of the plant like roots or leaves, stems, petioles, and flowers. All these parts or organs of the plant differ in morphology and function that means in their requirement to membrane transport of water or CO_2. In the root, water transport may be more important than gas transport and in leaves, CO_2 membrane transport is of major importance to support photosynthesis. A striking relevance of aquaporin expression could not only be shown for processes, which are obviously depending on water and CO_2, like root

water uptake or photosynthesis, but also for plant reproduction, leaf movements, symbiosis, and other processes. Analyzing the role of aquaporins throughout the plant appeared that in different organs often the same type of aquaporins performs different tasks. It seems that function of aquaporins is modified according to the requirements of the tissue or organ, where they are expressed.

3.1 Plant Reproduction

Processes during sexual reproduction and seed germination in higher plants involve the movement of water between cells or tissues. Water and nutrients needed to supply developing seeds are mainly supplied by the phloem and have to be released from a maternal parenchyma tissue before being utilized by the embryo and endosperm. Members of the aquaporin family were shown to be expressed in the developing pea seed coat (Schuurmans et al. 2003) and are likely to be involved in the cellular water transport.

Cellular water transport with relevance to plant reproduction also occurs during dehiscence of the anthers or hydration of pollen grains after they are deposited on the stigma. To get more insight into these processes, aquaporin expression in reproductive organs of tobacco was analyzed. It was found that at least 15 members from the PIP1 and PIP2 subfamily are expressed in reproductive organs, which indicates that the control of water flow is important for reproduction. Expression studies on RNA and protein levels showed that PIP1 and PIP2 genes are differently expressed in reproductive organs: PIP1 RNA accumulates in the stigma, and PIP1 and PIP2 RNA can be detected in most tissues of the anther (Bots et al. 2005a; Bots et al. 2005b).

3.2 Water Transport

Plant water balance is largely determined by the rate of root water uptake and radial transport into the xylem, its long distance transport through the xylem and finally the loss due to transpiration (Kaldenhoff et al. 2008). On long distances, water transport is not limited by membrane resistances. However, on short distances, which means during root water uptake, and radial water transport and non vascular paths, water has to cross cellular membranes. Aquaporin proteins have been shown to be located especially at these sites, e.g., in roots and stems surrounding the vascular tissues as well as in motor organs required for leaf movement (Moshelion et al. 2002; Otto and Kaldenhoff 2000).

Radial water transport in plant roots combines apoplastic and cell-to-cell pathways. It is a matter of ongoing discussion which component mainly determines hydraulic conductivity of plant roots. Aquaporins play an important role in

regulating the transcellular path of water transport. Steudle and coworkers analyzed the relative contribution of apoplastic and cell-to-cell paths to the overall hydraulic conductivity by partially blocking the porous apoplast with China ink particles (diameter 50 nm) or by inhibition of aquaporins in cell membranes with 50 μM $HgCl_2$ (Ranathunge et al. 2004). The reduction of hydraulic conductivity was relatively larger in the presence of an apoplastic blockage with ink (~30%) than in the presence of the water channel blocker (~10%). From these results, a relatively larger apoplastic water flow was deduced. However, the tools to inhibit water transport that were used follow highly different mechanisms: ink particles unspecifically block apoplastic regions and the application of mercurials affects a lot of other proteins besides aquaporins and thus, provokes plenty of other effects. In addition, the level of inhibition is not quantifiable in any case. None of both techniques can eliminate one component of water transport without affecting the other; in particular as both components depend on each other.

Different results were obtained via reverse genetic strategies. When the specific hydraulic conductivity of tobacco plants with reduced expression of NtAQP1 and respective control plants was analyzed, a strong correlation between cellular water permeability and specific root hydraulic conductivity was observed (Siefritz et al. 2002). This indicates that the transcellular pathway is very important for vascular and long distance water transport and that strictly apoplastic transport through cell walls is of minor impact, at least in roots.

3.3 Water Stress

The response of aquaporin expression to water stress can be diverse. It might involve either up or downregulation of gene expression or no change (Tyerman et al. 2002) depending on the time course and intensity of water stress (Galmes et al. 2007). The effect of abiotic stress on aquaporin expression was analyzed in olive trees, which were subjected to drought treatment (Secchi et al. 2007). The transcript levels of TIP1;1, PIP1;1 and PIP2;1 in *Olea europea* declined strongly in plants submitted to drought stress, when soil moisture, twig water potential, and twig hydraulic conductivity progressively decreased. Downregulation of aquaporin expression may result in reduced membrane water permeability and may limit loss of cellular water during periods of water stress.

3.4 Plant Movements and Rhythms

Contribution of aquaporins to plant movements and rhythms was reviewed (Uehlein and Kaldenhoff 2006, 2008). Plant leaf movements can be mediated in two different ways – reversible swelling of specialized motor cells or irreversible asymmetrical growth velocities of the adaxial and abaxial half of the leaf,

respectively. In contrast to typical growth movements, turgor movements are reversible. They were observed in many plants, like for example *Mimosa*, *Phaseolus*, *Albizzia*, *Desmodium,* and *Samanea*, which possess specialized motor organs, called pulvini. In contrast to typical growth movement, both processes are associated with diurnally regulated increased rates of membrane water transport, which in many cases was shown to be facilitated by aquaporins. Many details on architecture and function of pulvini initially were worked out by Ruth Satter and coworkers on *Samanea saman* as a model organism. More recently, a contribution of aquaporins to pulvinar movements in *Samanea* was shown (Moshelion et al. 2002). Another model plant to study pulvinus mediated leaf movements is *Mimosa pudica*. The contribution of both plasma membrane and tonoplast localized aquaporins to the seismonastic leaf movements was analyzed (Temmei et al. 2005; Fleurat-Lessard et al. 1997). Using tobacco as an example for epinastic leaf movement, it was shown that a PIP1 aquaporin family member is an important component of the leaf movement mechanism (Siefritz et al. 2004).

3.5 Defense Reaction

Cuscuta is an obligate stem parasite causing worldwide heavy losses in agriculture. *Cuscuta* has to establish a functional connection with its host plant to obtain photo-assimilates and water. A successful infection involves enzymatic mechanisms of cell wall loosening, mechanical penetration of host tissue, and establishment of haustoria. Finally, hyphae grow towards the hosts vascular tissues and connect to xylem and phloem, respectively. However, there are plants known, which possess defense mechanisms against an attack of *Cuscuta*, involving amongst others cell elongation of the epidermal cell layer. Tomato is such an incompatible host. One of the early induced genes during tomato–Cuscuta interaction encodes an aquaporin (LeAQP2), as it could be shown by sequence and functional analysis (Werner et al. 2001). The observed pattern of LeAqp2 expression during the interaction at a stage where cell elongation occurs together with the water-channel activity in the heterologous *Xenopus* oocyte expression system suggest a key function for LeAqp2 during the incompatible reaction of tomato upon attack by *Cuscuta*.

3.6 Photosynthesis

The resistance of biological membranes to gas transport was hardly recognized as a factor limiting metabolic function. However, and in contrast to the mammalian aquaporins (Cooper et al. 2002), a clear role of CO_2 conducting aquaporins in plant physiology could be shown. Tobacco plants with modified aquaporin expression were changed in water transport characteristics (Siefritz et al. 2002),

as well as in CO_2 dependent processes like rate of photosynthetic CO_2 fixation and stomatal movement (Uehlein et al. 2003). Also Aharon et al. (2003) reported a beneficial effect of aquaporin expression on stomata and photosynthesis. Under favorable growth conditions, tobacco plants overexpressing the aquaporin PIP1;2 from *Arabidopsis* showed a significant increase in transpiration rate and photosynthetic efficiency, as well as a higher stomatal density (Aharon et al. 2003). However, the authors had not analyzed the membrane transport of CO_2 and therefore, related the effects to an improved water transport. As CO_2 is the primary substrate for photosynthesis, its cellular uptake and transport is of fundamental importance for plants. To reach the place of CO_2 fixation in the chloroplast stroma, CO_2 has to cross a couple of membranes: the plasma membrane and at least two chloroplast envelope membranes. The tobacco plasma membrane and inner chloroplast membranes contain the aquaporin NtAQP1. An RNAi mediated decrease in NtAQP1 expression lowered the CO_2 permeability of the inner chloroplast membrane, but no significant effect could be shown for the plasma membrane. In vivo data showed that the reduced amount of NtAQP1 caused a reduction of CO_2 conductance within leaves and as a consequence, a reduced CO_2 fixation rate (Uehlein et al. 2008). Taken together, NtAQP1 acts as a CO_2 membrane transport facilitator and increases CO_2 transport at the inner chloroplast membrane, not at the level of the plasma membrane. Consequently, the inner chloroplast envelope membrane represents at least one of the main resistances for CO_2 conductance within leaves. However, there are yet more resistances to CO_2 transport. It has been shown that the cell walls constitute another major component of the liquid phase resistance (Terashima et al. 2006; Evans et al. 2009).

3.7 Symbioses: Mycorrhizas and Rhizobial Nodulation

Many terrestrial plants have developed a symbiosis with mycorrhizal fungi to improve plant nutrition under nutrient limited conditions and water uptake. Two major types of mycorrhiza are ectomycorrhizas and arbuscular endomycorrhizas. Establishment of the association is accompanied by structural and functional changes in the plant root.

During ectomycorrhizal symbiosis between poplar and *Amanita muscaria,* increased transcript levels of PIP1 and PIP2 were detected. In agreement with these findings, root hydraulic conductivity was increased under ectomycorrhized conditions (Marjanovic et al. 2005) resulting in an increased water transport capacity from the soil to the plant.

During arbuscule formation, fungal hyphae penetrate the root apoplast and install highly specialized interfaces for solute transport between plant and fungus. This is also the case in nodule formation during interaction of nitrogen fixing rhizobia and roots of legume plants, where the so called peribacteroid membrane is built. The periarbuscular membrane, which is part of the plasma membrane as

well as the peribacteroid membrane controls the direct fluxes of substances between plant and symbiont.

The role of aquaporins with regard to transport processes across these membranes can be quite numerous. The periarbuscular membrane was shown to contain a high density of different transport systems. Among these also expression of aquaporins was described. Aquaporins can potentially act as a transport system for ammonia or ammonium (Bertl and Kaldenhoff, 2007; Loque et al. 2005; Niemietz and Tyerman 2000). Indeed, the first plant aquaporins to be isolated and characterized were found in the peribacteroid membrane of nodulated soy bean roots, where high membrane fluxes of ammonia occur. Also, during mycorrhiza symbiosis significant nitrogen transfer in form of ammonia or ammonium from the fungus to the plant occurs (He et al. 2003). In endomycorrhized *Medicago truncatula* roots, upregulation of NIP and PIP2 aquaporins was shown. Functional analysis using heterologous expression systems revealed a strong water conductivity of MtPIP2;1 as well as a contribution of MtNIP1 to cellular uptake of ammonia (Uehlein et al. 2007).

3.8 Plant Nutrition

The effect of plant nutrition on the expression pattern of aquaporins in *Phaseolus vulgaris* plants was analyzed. Supplying plants with ammonium as exclusive nitrogen source causes negative effects on dry-mass formation, leaf growth, and leaf transpiration. The effect of N-form supply on root water uptake, leaf transpiration, and aquaporin expression was studied using a split root system (Guo et al. 2007). In ammonia supplied plants, root dry mass and water uptake rate were significantly reduced compared to nitrate supplied plants. An analysis of aquaporin expression showed that aquaporin expression on roots is lower under ammonium compared to nitrate treatment. From these data, the reduced water transport in ammonium treated compared to nitrate treated roots could be directly related to aquaporin expression. The control of aquaporin activity in this special case may be directly related to facilitation of NH_3/NH_4^+ membrane transport by aquaporins (Bertl and Kaldenhoff 2007; Jahn et al. 2004; Niemietz and Tyerman 2000). The suppression of aquaporin abundance under conditions of high external NH_4^+ supply may represent a direct control mechanism to reduce NH_4^+ uptake.

4 Importance of Plant Aquaporins

Aquaporins research in recent years has revolutionized the concept of membrane permeability to water, small neutral solutes, and gasses. The aforementioned collection of work focuses on functional characterization of plasma membrane intrinsic aquaporins delivering astonishing findings. Functional analysis of PIP1

aquaporins in heterologous expression systems showed that PIP1 increases water permeability of membranes only to a minor extent whereas the increase upon insertion of PIP2 proteins is very high (Bots et al. 2005a; Marjanovic et al. 2005; Moshelion et al. 2002; Secchi et al. 2007; Werner et al. 2001). It was described in the literature that PIP1 aquaporins are targeted to the plasma membrane only together with PIP2 aquaporins (Fetter et al. 2004; Zelazny et al. 2007). This may be one mechanism but the data here presented show that PIP1 also independently from PIP2 reaches the plasma membrane, where its function is clearly visible in terms of CO_2 conductivity (Uehlein et al. 2003). These and further data from heterologous expression show that, in principle, PIP2 aquaporins exhibit a high water conductivity but no conductivity to gasses. In contrast, PIP1 aquaporins exhibit a low water conductivity but considerable conductivity to CO_2 (Uehlein et al. 2003, 2008; Fischer 2007; Pede 2008; Fischer and Kaldenhoff, unpublished data). Hints are emerging that aquaporins may be involved in membrane transport of NH_3 in roots (Niemietz and Tyerman 2000; Uehlein et al. 2007; Guo et al. 2007).

Recent experiments have shown that the substrate specificity depends on the monomer composition within the aquaporin tetramers. Functional analysis of artificial heterotetramers with a defined ratio of PIP1 to PIP2 shows that appearance of PIP1 in heterotetramers favors gas conductivity while appearance of PIP2 favors water conductivity (Fischer and Kaldenhoff, unpublished data). This and of course the other concepts to regulate aquaporin activity, which were discussed before, can direct aquaporin-tetramer function to meet different tasks. Thus, PIP aquaporins can be regarded as a multicomponent system in membrane transport. This can explain how the same type of aquaporins can be involved in numerous functions throughout plant development and during adaptations to variable environmental conditions. The aquaporin PIP1;2, which was mainly analyzed in the present work is expressed in all parts of the plant (Otto and Kaldenhoff 2000), but in different organs of the plant different tasks are to be fulfilled. In the root, water transport is more important than gas transport, in leaves, membrane transport of CO_2 is of major importance to support photosynthesis. So, it is a matter of regulation and combining different attributes of different aquaporins to meet the cells requirement to water or CO_2 transport. Thus, NtAQP1 expressed in roots can be evidently involved into plant water relations (Siefritz et al. 2002). However, in leaves it switches its function to that of a CO_2 pore supporting photosynthesis (Uehlein et al. 2003, 2008). The requirements to membrane water and CO_2 permeability in the plant differs on tissue or organ level and it is obvious that the function of PIP1 aquaporins is modified according to the respective requirements. In leaves, some PIP1 proteins might primarily act as transporters for small solutes or gasses and in roots, they could switch to water channel activity triggered by modifications or interaction with other aquaporins. In motor organs like pulvini and petioles, PIP1 aquaporins are involved in proper functioning of the leaf movement mechanism (Moshelion et al. 2002; Siefritz et al. 2004; Uehlein and Kaldenhoff 2006). Due to the high membrane water permeability obtained in different heterologous expression systems, PIP2 aquaporins may represent the major pathways for short distance water transport in roots and petioles.

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Systematics

Lichen Systematics: The Role of Morphological and Molecular Data to Reconstruct Phylogenetic Relationships

Christian Printzen

Contents

1 Introduction

It took almost a century until Schwendener's (1867) finding that lichens belong to the fungi finally led mycologists and lichenologists to include them in the fungal system (Nannfeldt 1932; Santesson 1952). Trying to elucidate the phylogenetic relationships between lichenized and un-lichenized fungi and among lichen

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taxa, based solely on morphological and chemical data, has proven to be a frustrating endeavour. Lichens display few taxonomically useful characters, of which many are widely variable; the homology of character states within and between groups is difficult to assess. Often, even the interpretation of morphological characters, e.g. types of ascoma development or ascus type, proved difficult (see e.g. Henssen and Jahns 1974; Lumbsch 2000; Lumbsch et al. 2001c; Ott and Lumbsch 2001; Stenroos et al. 2002b; Lumbsch and Huhndorf 2007). In the absence of well-supported and uncontroversial phylogenetic reconstructions based on morphological data, molecular data have, therefore, gained great importance in lichen systematics. The impact of molecular data on the classification and taxonomy of lichenized ascomycetes has been summarized regularly in recent years (Lumbsch 2000, 2007; Grube and Winka 2002; DePriest 2004). This review is not an attempt to update these previous comprehensive reviews. It rather tries to shed light on the relationship between results based on molecular and morphological studies of lichens. In the late 1980s and early 1990s, morphology-based taxonomy and systematics and molecular phylogenetics of lichens more or less led their own separate lives. The first studies based on molecular data often concentrated on reconstructing phylogenetic relationships and were not so much concerned with character evolution or the reinterpretation of morphological characters in light of molecular results. Likewise, a critical evaluation of the results in light of morphological data was rarely attempted. This has changed profoundly in recent years. Most phylogenetic reconstructions of lichenized ascomycetes are now designed to test morphology-based classifications. As a result, the systematic value of morphological characters in diverse groups is now much better understood than previously and reconstructions of character evolution exist for many systematic groups. On the other hand, classical taxonomists make increasing use of molecular data because classical lichen taxonomy is riddled with problems that only independent data from molecular analyses are likely to solve. One very obvious problem that is relatively easy to solve with molecular data concerns the systematic placement of obligately sterile lichens (Stenroos and DePriest 1998; Arup and Grube 1999; Platt and Spatafora 2000; Ekman and Tønsberg 2002; Crespo et al. 2004a) or other species with doubtful systematic affinities (Printzen and Kantvilas 2004; Lücking et al. 2007; Spribille et al. 2009). Other such problems arise from the multiple description of morphologically variable species, doubtful circumscriptions of taxa and erroneous assignment of species to them, or misinterpretation of the systematic value of characters due to incorrect homology hypotheses. In all these cases, molecular analyses offer promising tools to test traditional hypotheses.

This is not to say that molecular data can replace careful morphological investigations. It is not just morphology-based hypotheses that need testing by independent, molecular data. The opposite is equally true. It is the rule rather than the exception that traditional systematic concepts based on morphological characters are in conflict with molecular reconstructions. This may be due to insufficiencies in traditional concepts, but molecular results also have to be treated with care. Well-known problems with molecular phylogenetic reconstructions include: (1) use

of erroneous sequences, e.g. from misidentifed voucher specimens or unrecognized parasymbionts (e.g. Zhou et al. 2006; Reese Næsborg et al. 2007); (2) poor taxon sampling that may affect monophyly hypotheses and the reconstruction of phylogenetic relationships (Wedin and Wiklund 2004; Tehler and Irestedt 2007); and (3) incongruence between gene genealogies and species phylogenies and problems recovering the correct phylogeny from a limited set of genes (Aguileta et al. 2008). A critical interpretation of molecular results entails a sound knowledge of the anatomy, morphology, and secondary chemistry of the taxonomic groups under study. Even traits that have been used in fungal systematics for decades have often not been studied in certain groups or have been misinterpreted. For example, Liew et al. (2000) interpreted the Chaetothyriomycetidae as an ascolocular group, while in fact many lichenized taxa show an ascohymenial ontogeny.

Due to the variability and paucity of useful morphological characters in lichens, few cladistic analyses based on phenotypic data have been published (Grube 1998; Nordin and Mattsson 2001; Aptroot and Lücking 2003; Lücking et al. 2005; Lumbsch and Tehler 1998; Tehler 1990, 1995a, 1995b). It is therefore not possible to simply compare phylogenetic trees from phenotypic and molecular data when trying to assess the degree of conflict between morphological and molecular results. A character based approach is therefore often attempted in which traditional hypotheses on the homology of certain characters are confronted with the molecular evidence. As long as the phylogenetic position of many groups is not known with sufficient certainty, reconciling molecular and morphology-based concepts remains an iterative process.

This chapter begins with a brief outline of systematically important characters and reviews what molecular studies have contributed to their interpretation. The contributions of molecular studies to the systematics and classification of lichens since approximately the year 2000, starting with higher-level relationships and continuing down to the family and genus levels, is summarized. In addition to morphological features, chemical and anatomical characters have played important roles in lichen systematics. For simplicity, the terms “morphology” and “morphological” are used to include anatomical and chemical characters throughout this text.

2 Characters

2.1 Ascoma Type and Ontogeny

Ascoma type and development are among the key characters used to distinguish higher taxa within the ascomycetes. Some monophyletic groups may indeed be circumscribed by a certain ascoma type. For example, all unlichenized ascomycetes with perithecia belong to the monophyletic Sordariomycetes (Lumbsch et al. 2005a) and, within the Dothideomycetes, ascoma centrum and ontogeny are important characters for the recognition of major clades (Lumbsch and Huhndorf 2007). Lichens with perithecia, however, do not form a monophyletic group but are

distributed across various classes (Dothideomycetes, Eurotiomycetes, Lecanoromycetes) (Lumbsch and Huhndorf 2007). The molecular evidence gathered up to now indicates that apothecia are plesiomorphic within the filamentous ascomycetes and that pseudothecia and cleistothecia are homoplasious (Lumbsch 2000; Lumbsch et al. 2002). Within the Lecanoromycetes, perithecioid and apothecioid ascomata can be found within the same orders or families (e.g. *Porina* and *Belonia* in the Ostropales). Molecular analyses have recently contributed to the interpretation of ascoma types. In the Coccotremataceae, ascomata were either interpreted as apothecia or perithecia. Molecular data allowed the conclusion that they are in fact hemiangiocarpic apothecia (Lumbsch et al. 2001c). Likewise, the perithecia found in the Porinaceae may in fact be neotenic hemiangiocarpic apothecia (Grube et al. 2004a). The example of the "order" Caliciales nicely illustrates how similarities in ascoma morphology can be misleading when trying to circumscribe higher lichen taxa. Caliciales seemed to be well characterized by mostly stalked apothecia with prototunicate asci forming a mazaedium. However, the polyphyly of the group was demonstrated frequently (Wedin and Tibell 1997; Tibell and Wedin 2000; Wedin et al. 2000b; Lumbsch et al. 2004a, 2009). Whether ascolocular and ascohymenial ontogeny circumscribe monophyletic groups of ascomycetes is still a bit uncertain. The ascohymenial type appears to be plesiomorphic (Lumbsch et al. 2002), but Loculoascomycetes are either reconstructed as monophyletic (Liew et al. 2000; Liu and Hall 2004) or polyphyletic (Lindemuth et al. 2001; Lumbsch et al. 2000, 2002). At present, it thus seems that neither ascoma type nor ontogeny can be used to define any major lineages of ascomycetes (Lutzoni et al. 2004).

2.2 Mode of Reproduction

The reproductive mode has been used to distinguish taxa from each other, but almost exclusively at the species level. In so-called "species pairs" (for a discussion see Tehler 1982; Mattsson and Lumbsch 1989), one of two or three morphologically similar species propagates sexually, while the other(s) use(s) different means of vegetative propagation. Molecular data shows that these presumed species are often not reciprocally monophyletic (Lohtander et al. 1998a, 1998b; Myllys et al. 1999b, 2001; Articus et al. 2002; Molina et al. 2002; Ott et al. 2004; Tehler et al. 2004; Buschbom and Barker 2006; Buschbom and Mueller 2006). But in other cases, monophyletic species have been recovered (Tehler and Källersjö 2001) or additional characters have been found to separate lineages within apparently polyphyletic species (Wirtz et al. 2006).

2.3 Ascus Characters

The systematic value of ascus anatomy has long been discussed. Recent molecular analyses have demonstrated that operculate asci evolved once in the Pezizomycetes

and inoperculate asci twice in the Orbiliomycetes and Leotiomycetae (Tehler et al. 2003; Spatafora et al. 2006; Hibbett et al. 2007). Hence, operculate and inoperculate asci characterize large monophyletic lineages within the ascomycetes (Lutzoni et al. 2004). Other ascus types, however, do not seem to characterize phylogenetic groups. Although the majority of ascomycetes with unitunicate asci are combined in a single clade (Sordariomycetes and Leotiomycetes) (Spatafora et al. 2006), the various types of inoperculate asci (prototunicate, unitunicate and bitunicate) appear to be highly homoplasious (Lumbsch 2000; Lumbsch et al. 2002). Phylogenetic groups based on this character alone are mostly polyphyletic (Grube and Hawksworth 2007). Prototunicate asci are, for example, found in the Lichinomycetes, Arthoniomycetes and Lecanoromycetes (Ostropomycetidae and Caliciaceae). However, differences in ascus anatomy coincide with the two largest subclasses of Lecanoromycetes: the Ostropomycetidae with weakly amyloid and the Lecanoromycetidae with mostly strongly amyloid asci (Persoh et al. 2004; Lumbsch et al. 2007a). Within the Ostropomycetidae, ascus types are apparently of little taxonomic use (Lumbsch et al. 2001b, 2007b).

Since the early 1980s, ascus apical structures have gained enormous importance for the delimitation and classification of Lecanoromycetes (Hafellner 1984). The subordinal classification of Lecanorales suggested by Rambold and Triebel (1992) was mainly based on similar ascus "types". Other authors pointed out a relatively high variability of ascus tips within genera (e.g. Hertel and Rambold 1985) that made it often impossible to assign asci to a certain type (e.g. Hinteregger 1994, p. 95, Hertel and Rambold 1995; Printzen 1995, p. 48). The ascus types used to distinguish the two families Lecideaceae and Porpidiaceae proved to be modifications of the same basic type (Buschbom and Mueller 2004). Unfortunately, one gets stuck in circular reasoning when trying to estimate the variability of a character in supposedly closely related groups that are defined based on this same character. For example, Hafellner (1984) distinguished several families around *Lecanoraceae* and *Bacidiaceae* based on slight differences in ascus amyloid reactions, but Hertel and Rambold (1995) suggested that the ascus types of both families were just modifications of a more variable type and suggested to synonymize them. Molecular studies show that each of the two families is monophyletic but both are relatively remotely related (Miadlikowska et al. 2006). Deviating ascus types occur in each of them (Ekman et al. 2008). Anatomically similar asci occur in unrelated groups of Lecanoromycetes, and families or genera that were assigned to different suborders based on their ascus types may in fact be closely related. *Lecanora*-type asci, for example, occur in *Lecanora* (Lecanoraceae), *Rinodina* (Physciaceae, Rambold et al. 1994) and *Pleopsidium* (Acarosporaceae), while the closely related families Ramalinaceae and Pilocarpaceae (Andersen and Ekman 2005) display ascus types that were formerly used to characterize the suborders Lecanorineae and Cladoniineae. Polyspory has evolved at least four times independently within the Lecanoromycetes (Reeb et al. 2004). On the other hand, the number of spores per ascus may be a good character to separate species, as in *Lecanora cyrtella* and *L. sambucina* (Reese Næsborg et al. 2007).

2.4 Ascospores

Since the middle of the nineteenth century, ascospore morphology has developed into one of the prime characters used to distinguish genera and families in many groups of fungi, including lichens (Körber 1855, 1865; Vainio 1890; Zahlbruckner 1926). Although spore characters are usually more or less homogeneous within genera (e.g. pluriseptate, elongated, un-pigmented spores in *Bacidia*, or large, non-septate and thick-walled spores in *Pertusaria*), molecular evidence indicates that most types have evolved several times independently within the Lecanoromycetes or euascomycetes in general and constitute a highly homoplasious character (Ihlen and Ekman 2002; Helms et al. 2003; del Prado et al. 2006; Frisch et al. 2006; Lumbsch et al. 2006; Miadlikowska et al. 2006; Staiger et al. 2006; Gueidan et al. 2007; Savić et al. 2008). Hence, ascospore characters usually do not reflect phylogenetic relationships, although they may circumscribe certain monophyletic groups. The Physciaceae and Teloschistaceae are an interesting exception. Both families comprise species with characteristically thick-walled, usually one-septate (so-called polar diblastic) ascospores, which are typically brown in the Physciaceae and colourless in the Teloschistaceae. The striking similarities in ascospore anatomy already led lichenologists in the nineteenth and early twentieth century to treat these families (or the genera *Physcia* and *Xanthoria*) as synonymous (Körber 1855; Nylander 1854) or regard them as closely related (Lynge 1916); molecular data support such a close relationship.

2.5 Thallus Organization

In traditional lichen systems, genera and families were often delimited based on thallus growth form. More recent studies, however, indicate that this character is highly variable and that crustose, foliose, fruticose and calicioid lichens can occur within one and the same family (Arup and Grube 1999, 2000; Myllys et al. 1999a; Grube and Winka 2002; Wedin et al. 2002; Gaya et al. 2003, 2008; Helms et al. 2003; Tehler and Irestedt 2007; Westberg et al. 2007). Cladoniiform lichens evolved several times independently (Stenroos and DePriest 1998). Even ontogenetic characters can sometimes be misleading. Neither the presence of podetia in Cladoniaceae nor that of pseudopodetia in Stereocaulaceae have proven to be synapomorphous (Wedin et al. 2000a). The occurrence of specialized vegetative structures, such as cyphellae and pseudocyphellae, also does not indicate monophyly (Thomas et al. 2002; Stenroos et al. 2003).

The association with certain photobionts has often been used to circumscribe taxa of lichenized ascomycetes. This character has frequently been demonstrated to be homoplasious, especially within the Ostropomycetidea (Wedin et al. 2000a; Lumbsch et al. 2001c; Schmitt et al. 2003a) and the Peltigerales (Ekman and Jørgensen 2002; Lohtander et al. 2002; Miadlikowska et al. 2003). However, phylogenetic lineages may also display specific photobiont selectivity, e.g. *Diploschistes* within the Thelotremataceae (Martín et al. 2003). Higher-level

phylogenies clearly show independent gains and losses of lichenization and association with certain photobionts (Lutzoni et al. 2001; Högnabba et al. 2009). The number of studies dealing with photobiont selectivity and specificity of lichens has greatly increased recently (e.g. Muggia et al. 2008a) but results from these studies have little taxonomic consequence and hence will not be reviewed here.

2.6 Secondary Metabolites

Most lichen taxa produce secondary metabolites in considerable numbers and amounts. Because morphological characters used to distinguish lichens are often scarce or too variable, chemical differences are used to delimit taxa and identify otherwise similar species. Especially within sterile groups, such as *Lepraria*, secondary compounds have become important characters to distinguish species (Fehrer et al. 2008). Before molecular data provided independent evidence, it was difficult to assess whether chemical differences reflected infraspecific variability or isolation of phylogenetic lineages (see Hawksworth 1976; Lumbsch 1998). The genetic background of chemical variation between and within lichen taxa is still poorly understood, because the polyketide synthase genes that catalyse important steps in the biosynthesis of most secondary lichen compounds have only just begun to be studied (Grube and Blaha 2003; Opanowicz et al. 2006; Muggia et al. 2008b; Schmitt et al. 2008; Stocker-Wörgötter 2008; Schmitt and Lumbsch 2009; Brunauer et al. 2009; Gagunashvili et al. 2009). It is interesting to note that polyketide metabolites occur in lichenized and secondarily de-lichenized, but not in primarily non-lichenized, ascomycetes (Grube and Winka 2002). The expression of these metabolites is apparently restricted to phylogenetically, rather than ecologically, circumscribed groups. The widespread (and often sporadic) occurrence of most secondary metabolites across the lichenized ascomycetes indicates that they convey little information on phylogenetic relationships, although some have proved useful when distinguishing species (LaGreca 1999; Kroken and Taylor 2001; Tehler and Källersjö 2001; Lumbsch et al. 2007c, 2008c; Fehrer et al. 2008; Lücking et al. 2008a) or even higher-level monophyletic groups. For example, the occurrence of picrolichenic acid is restricted to the *Variolaria*- group of the Pertusariaceae. Other clades of this family are also well characterized by chemical compounds (Schmitt and Lumbsch 2004), and the separation of Baeomycetaceae and Icmadophilaceae is supported by chemical characters (Platt and Spatafora 2000). In other cases, however, secondary metabolites do not characterize monophyletic groups (Buschbom and Mueller 2006).

3 Lichens and the Phylogeny of Ascomycetes

Because lichens are not an evolutionary but a nutritional group of fungi, their higher-level classification cannot be separated from that of unlichenized fungi. The first molecular studies on the higher-level phylogeny of fungi that also included

lichens were conducted in the early 1990s. The results of these early studies have been reviewed elsewhere (Lumbsch 2000; DePriest 2004). Not surprisingly, molecular data supported the view that lichenization had evolved several times in different groups of fungi (Gargas et al. 1995). More recent results by Lutzoni et al. (2001) indicate that a loss of lichenization occurred as well in several major lichenized ascomycete clades. Such conclusions rely heavily on statistically well-supported phylogenetic reconstructions. Unfortunately, statistical support, especially in the backbone of fungal phylogenies, is still a major problem and an indicator that the molecular data accumulated so far is not sufficient to resolve the early radiations within the major fungal groups (Berbee et al. 2000). Whether or not a broader taxon sampling can resolve the problem is not well understood at present. The results of Lumbsch et al. (2007a) or Miadlikowska et al. (2006) indicate that the exclusion of taxa may have an impact on the ingroup rooting. As long as there is no firm evidence for the monophyly of many higher taxa, it is certainly necessary to include more than just a handful representatives of these taxa to resolve their sister group relationships.

Early phylogenetic studies were based on single genes, usually included only few taxa, and often came to contradictory conclusions. Based on nSSU sequences, Spatafora (1995) reconstructed the "Pyrenomycetes" (i.e. Sordariales, Hypocreales, Microascales and Diaporthales) together with Pleosporales as basal to the rest of the filamentous ascomycetes. On the other hand, the phylogenetic tree presented by Lumbsch (2000) shows Pezizomycetes at the base of the filamentous ascomycetes, a position that was later confirmed by many other authors, mostly as a monophyletic group (Bhattacharya et al. 2000, 2005; Grube and Winka 2002; Liu and Hall 2004; Lutzoni et al. 2004; Spatafora et al. 2006) but sometimes also as a paraphyletic one (Lutzoni et al. 2004; Persoh et al. 2004; Reeb et al. 2004). Most molecular studies also agree that the pyrenomycetes (ascomycetes forming perithecia) are not monophyletic (Lumbsch and Huhndorf 2007). The Arthoniomycetes appear as sister group to the Sordariomycetes (Lumbsch 2000; Bhattacharya et al. 2000, 2005; Lutzoni et al. 2001; Grube and Winka 2002) but monophyletic clades of Leotiomycetes and Sordariomycetes (Lumbsch et al. 2002, 2005a; Liu and Hall 2004; Spatafora et al. 2006), and Dothideomycetes and Arthoniomycetes (Lutzoni et al. 2004; Lumbsch et al. 2005a; Geiser et al. 2006; Spatafora et al. 2006; Hibbett et al. 2007) were also reconstructed. The phylogenetic position of the Dothideomycetes seems especially uncertain. In phylogenetic trees, members of the group appear to be related to Sordariomycetes and/or Arthoniomycetes (Lumbsch 2000; Lutzoni et al. 2004; Bhattacharya et al. 2005; Geiser et al. 2006; James et al. 2006; Spatafora et al. 2006), paraphyletic at the base of the Lecanoromycetes or basal to Lecanoromycetes, Chaetothyriomycetidae and Eurotiomycetidae (Grube and Winka 2002; Schmitt et al. 2005), basal to the inoperculate ascomycetes (Lumbsch et al. 2002; James et al. 2006) or sister to the Chaetothyriomycetidae (Liu and Hall 2004; Persoh et al. 2004). In most studies, the Chaetothyriomycetidae are grouped together with the Eurotiomycetidae (but see Liu and Hall 2004) and both are mostly combined under the Eurotiomycetes, which are either sister to the Lecanoromycetes

(Bhattacharya et al. 2000, 2005; Lumbsch 2000; Lumbsch et al. 2002, 2004b; Lutzoni et al. 2004) or nested within this class (Lutzoni et al. 2001; Grube and Winka 2002; Kauff and Lutzoni 2002; Persoh et al. 2004; Wedin et al. 2005a). Four- or five-gene phylogenies inferred a position sister to Mycocaliciales (Geiser et al. 2006), basal to a clade comprised of Lichinomycetes, Lecanoromycetes, Leotiomycetes and Sordariomycetes (Spatafora et al. 2006) or basal within the Leotiomycetae (Lumbsch et al. 2005a), although with poor statistical support. From a lichenological point of view, the sister group relationships of Lecanoromycetes, the class comprising most lichenized fungi, are of special interest. Most studies that indicated the Eurotiomycetes as closest relatives of the Lecanoromycetes did not include many representatives of the Lichinomycetes and Leotiomycetes. A more comprehensive taxon sampling seems to indicate that these two classes are in fact the closest relatives of Lecanoromycetes (Miadlikowska et al. 2006; Spatafora et al. 2006; Hofstetter et al. 2007).

These uncertainties about phylogenetic relationships among major groups of ascomycetes make it difficult to infer the systematic value or evolutionary history of the major characters listed see above. But interpreting the phylogenetic history and taxonomic importance of morphological characters is not only impeded by uncertainties about the phylogenetic relationships of major groups. A lack of knowledge about basic morphological characters in certain groups of fungi is also apparent. For example, Liu and Hall (2004) concluded that ascolocular ascomata are derived and evolved only once, while Lumbsch et al. (2005a) point out that some of the groups thought to show an ascolocular development (Arthoniomycetes, Verrucariales) display in fact an ascohymenial ontogeny. Schmitt et al. (2005) warn against the uncritical interpretation of ascus types because groups traditionally treated as bitunicate, such as *Thrombium* and the Gomphillaceae, have recently been shown to have functionally unitunicate asci.

4 Systematics of Lichenized Ascomycota

4.1 Arthoniomycetes

The closely related orders Arthoniales and Opegraphales were combined in the class Arthoniomycetes (Eriksson and Winka 1997). The Arthoniales originally comprised lichenized fungi with bitunicate asci and an ascoma ontogeny that was alternatively regarded as ascolocular or ascohymenial (see above). Most species are associated with trentepohlioid green algae. The four calicioid genera *Sporostigma, Tylophoron, Tylophorella* and *Wegea* were recently transferred to the Arthoniaceae (Lumbsch et al. 2009), thus providing further evidence for the multiple evolution of prototunicate asci. So far, most higher-level phylogenetic studies have included no or only very few Arthoniomycetes (e.g. Lutzoni et al. 2001; Schultz et al. 2001;

Reeb et al. 2004; Spatafora et al. 2006; Lücking et al. 2008b). Molecular results indicate that the separation of Opegraphales and Arthoniales cannot be upheld. Ertz et al. (2009) found the genus *Opegrapha* to be highly polyphyletic. The *O. varia* group appears as a sister group to the Arthoniaceae, *O. atra* and *O. calcarea* nested within *Arthonia*. Other species are closely related to species of the polyphyletic genus *Enterographa* and other Roccellaceae. The development of a prominent and carbonized exciple used to define some genera within the Arthoniales does not define phylogenetic groups. Another character that proved to be homoplasious within the Arthoniomycetes is thallus growth form, a key character for the delimitation of genera within the Roccellaceae. The majority of fruticose *Roccella*-species are sisters to the crustose genus *Dirina*, but four species are grouped within *Roccellina* (Myllys et al. 1999a; Tehler and Irestedt 2007), which leaves this genus paraphyletic. A re-defined genus *Roccellina* is left without morphological apomorphies (Tehler and Irestedt 2007). However, hypothecial pigmentation coincides with two major clades within the family. Differences in reproductive strategy (sexual vs. asexual by soredia) that were used to separate several morphologically similar pairs of species of Roccellaceae are not supported by molecular data (Lohtander et al. 1998a, 1998b; Myllys et al. 1999b; Tehler et al. 2004). It has long been pointed out that phylogenetic reconstructions of the Roccellaceae based on morphological data (e.g. Tehler 1990, 1995a, 1995b) are in conflict with molecular data (Myllys et al. 1998). Nevertheless, most species and even clades within the genus could be distinguished by morphological characters in a study on Mediterranean and Macaronesian *Roccella*-species (Tehler et al. 2004).

4.2 Dothideomycetes

The ascolocular Dothideomycetes comprise mainly non-lichenized fungi, but three families contain lichenized species (Arthopyreniaceae, Trypetheliaceae and Dacampiaceae). Muggia et al. (2008c) reported that the filamentous lichens *Cystocoleus* and *Racodium* belong in the otherwise pathogenic Capnodiales and suggested that they might also occur in the non-lichenized state. Based on the occurrence of bitunicate asci and similarities in ascospore anatomy, the Trypetheliaceae were traditionally placed close to the Pyrenulaceae (Chaetothyriomycetes). Molecular data place the Trypetheliaceae within the Dothideomycetes (Lutzoni et al. 2004; del Prado et al. 2006; Spatafora et al. 2006) or together with the Arthoniomycetes as a sister group to the Dothideomycetes (Schoch et al. 2006). This last result may be a result of poor taxon sampling. Del Prado et al. (2006) and Kruys et al. (2006) confirmed the placement of Arthopyreniaceae within Dothideomycetes. The generic delimitations within Trypetheliaceae, based on ascospore and perithecial characters, do not reflect phylogenetic relationships (del Prado et al. 2006). Lücking et al. (2007) found the enigmatic *Thelenella terricola* to belong to the Trypetheliaceae and described the new genus *Aptrootia* to accommodate it.

4.3 *Eurotiomycetes*

Several studies show that the Verrucariales and Pyrenulales belong to the Eurotiomycetes (Lutzoni et al. 2001, 2004; Lumbsch et al. 2004b; Schmitt et al. 2005; del Prado et al. 2006). The monophyletic Chaetothyriales and Verrucariales form a well-supported sister group to the Pyrenulales in recent molecular analyses (Geiser et al. 2006; James et al. 2006).

The Pyrenulales are mostly lichenized, tropical fungi and have been studied only superficially. The lichenized members are associated with trentepohlioid green algae and are exclusively crustose. Results from del Prado et al. (2006) indicate that the genera *Pyrenula* and *Celothelium* are monophyletic. The prototunicate genus *Pyrgillus* was recently transferred to the Pyrenulaceae (Lumbsch et al. 2004a). Morphologically based genus concepts have not yet been compared with molecular data.

The lichenized Verrucariaceae encompass umbilicate, squamulose and crustose members that have developed symbioses with a wide array of photobionts. Thallus growth form, the presence or absence of hymenial algae, and ascospore septation were traditionally used to distinguish genera. Apart from some smaller genera that appear to be monophyletic (e.g. *Bagliettoa, Catapyrenium, Dermatocarpon, Endocarpon*), generic delimitations within the Verrucariaceae do not reflect phylogenetic groups (Gueidan et al. 2007, 2009). *Verrucaria, Staurothele* and *Thelidium* are polyphyletic. However, certain morphological traits, such as pycnidium or cortex type, seem to characterize monophyletic groups within the Verrucariaceae. Savić et al. (2008) recovered the same monophyletic groups with somewhat different relationships, but support for interior nodes is low in both analyses. Different cortex types seem to be associated with endolithic, crustose and squamulose growth forms, although, in general, growth form seems to be highly homoplasious within the Verrucariaceae (Gueidan et al. 2009; Orange 2009). Simple ascospores are plesiomorphic within the Verrucariaceae, and although spore septation seems to be homoplasious, three well-supported clades characterized by muriform ascospores were recovered by Savić et al. (2008). Interestingly, the presence of hymenial algae, though also homoplasious, was inevitably correlated with the occurrence of muriform ascospores (Gueidan et al. 2007). The development of the involucrellum seems to be of some taxonomic significance in *Thelidium* (Thüs and Nascimbene 2008). Currently, it seems as if most morphological characters are homoplasious within the crustose Verrucariaceae and as if monophyletic groups are not well distinguished by morphological characters (Savić 2007; Savić and Tibell 2008). Especially the largest genus of the family, *Verrucaria*, appeared to be mainly characterized by plesiomorphic characters and has been split into several resurrected or newly described genera (Gueidan et al. 2009). The foliose *Dermatocarpon* was supposed to be closely related to the squamulose genus *Catapyrenium* by Heiðmarsson (2003) but appeared to be more closely related to either *Endocarpon*, with which it was more or less synonymous in the nineteenth century (Gueidan et al. 2007), or *Placidium* and allied genera (Savić et al. 2008). Many taxa within the *Dermatocarpon miniatum* group are polyphyletic, but ascospore length, the reaction with Melzer's reagent and type of epinecral layer

seem to coincide with some clades (Heiðmarsson 2003). Based on a larger taxon sample, Amtoft et al. (2008) recovered several morphologically well-distinguished species around *D. miniatum*, but *D. americanum* remained polyphyletic.

4.4 *Lecanoromycetes*

The overwhelming majority of all lichenized fungi belongs in the Lecanoromycetes. The phylogenetic relationships of orders within the Lecanoromycetes are still relatively unclear. Early molecular studies indicated that the suborders Acarosporineae, Pertusariineae and Umbilicarineae had to be excluded from the large order Lecanorales, while the Teloschistineae and Agyriineae fell within this group (Stenroos and DePriest 1998). Later analyses indicated that Ostropales, Agyriales and Pertusariales s. lat. form a monophyletic group that is embedded in a widely circumscribed Lecanorales s. lat. (Wedin et al. 2005a; Miadlikowska and Lutzoni 2004; Lumbsch et al. 2001a, 2007a). However, different relationships between these orders have been reconstructed as well (e.g. Kauff and Lutzoni 2002; Stenroos et al. 2002a; Grube et al. 2004a; Lücking et al. 2004). Practically all analyses agree that the Lecanorales s. lat. (including Umbilicariaceae, Candelariaceae, Acarosporaceae, but excluding Teloschistales and Peltigerales) are polyphyletic or paraphyletic. The most recent multigene phylogenies seem to lead to some stability regarding monophyletic groups (Miadlikowska et al. 2006). Until shortly before molecular data became available, the delimitation of suborders within the largest order Lecanorales was mainly based on ascus characters (Hafellner 1988; Rambold and Triebel 1992). Molecular data convincingly demonstrated that some of these suborders were polyphyletic and that orders, such as the Teloschistales or parts of the polyphyletic Caliciales in fact belonged in the Lecanorales (Stenroos and DePriest 1998; Wedin et al. 1998).

Using Leotiomycetes and Lichinomycetes as an outgroup, Hofstetter et al. (2007) reconstructed a five-gene phylogeny of the Lecanoromycetes in which the five subclasses Lecanoromycetidae, Ostropomycetidae, Umbilicariomycetidae, Acarosporomycetidae and Candelariomycetidae were supported as monophyletic, the last two being basal to the rest of the Lecanoromycetes.

4.4.1 Acarosporomycetidae

The phylogenetic relationships of the Acarosporaceae are still somewhat uncertain. Their position within the Lecanorales was not doubted, but because of their ascus type, they were kept in the suborder Acarosporineae for some time (Rambold and Triebel 1992). It is now clear that they fall outside the two major clades of Lecanoromycetes, the Lecanoromycetidae and Ostropomycetidae (Stenroos and DePriest 1998; Lutzoni et al. 2001; Miadlikowska and Lutzoni 2004). Their position is probably at the base of Lecanoromycetes (Miadlikowska et al. 2006; Lumbsch et al. 2008c). However, Wedin

et al. (2005a) and Lücking et al. (2008b) inferred a closer relationship with Eurotiomycetes, although with poor support. Reeb et al. (2004) described the subclass Acarosporomycetidae. The polysporous genera *Pleopsidium* and *Timdalia* were transferred to the Lecanoraceae on account of their *Lecanora*-type asci, but in fact belong to the Acarosporaceae (Reeb et al. 2004; Wedin et al. 2005a).

The Acarosporaceae were chiefly characterized by polysporous asci but the study by Reeb et al. (2004) revealed that true polyspory has evolved at least four times (in *Sporastatia, Strangospora, Thelocarpon* and *Acarospora*) and that the genera *Biatorella, Hymenelia, Maronea, Sarcosagium, Sporastatia, Strangospora* and *Thelocarpon* do not belong into the Acarosporaceae. Generic delimitations within Acarosporaceae were based on exciple pigmentation and paraphysis anatomy, but are in need of revision. Neither *Acarospora* nor *Pleopsidium, Polysporinopsis* or *Sarcogyne* were monophyletic in recent analyses (Reeb et al. 2004; Crewe et al. 2006). Because the morphological variability within the large genus *Acarospora* is still poorly understood, species delimitations are uncertain. Wedin et al. (2009) found seven phylogenetic species within the *A. smaragdula* complex, of which most were morphologically well characterized.

4.4.2 Candelariomycetidae

The family Candelariaceae has been regarded as closely related to the Lecanoraceae because of its similar ascus type and the lecanorine amphithecium, but kept distinct due to its particular secondary chemistry of pulvinic acid derivatives. A position within the Lecanoraceae was inferred by LaGreca and Lumbsch (2001b) based on molecular, anatomical and ontogenetic data. However, the family has meanwhile proven to be basal to the rest of the Lecanoromycetes (Wedin et al. 2005b; Miadlikowska et al. 2006; Lumbsch et al. 2008c), but it is unclear whether it is outside or inside the Acarosporomycetidae. Miadlikowska et al. (2006) tentatively referred them to the subclass Candelariomycetidae. In a recent analysis, *Candelariella* appears paraphyletic and *Candelaria* polyphyletic, indicating that thallus morphology does not reflect phylogenetic relationships within the family (Westberg et al. 2007). Although several morphologically similar species pairs were described that differed mainly in the number of spores per ascus, polysporous species form several monophyletic groups. However, because of low statistical support in the backbone of the tree, it cannot be ruled out that polyspory arose only once within the Candelariaceae.

4.4.3 Ostropomycetidae

The order Ostropales originally comprised the non-lichenized Stictidaceae and related genera, but molecular results have placed an ever larger number of families close to Ostropales (e.g. Agyriaceae, Gomphillaceae, Graphidaceae, Icmadophilaceae, Pertusariaceae, Porinaceae). The subclass Ostropomycetidae has been proposed to

accomodate these groups. Phylogenetic relationships within Ostropomycetidae are far from being resolved. Not only genera have been shuffled around among families, but also families among orders. For example, Lumbsch et al. (2004b) suggested a distinction between Graphidales (*Graphis, Diploschistes, Thelotrema*) and Ostropales s. str. (*Absconditella, Stictis, Conotrema, Bryophagus*). In their analysis, these two orders form a strongly supported monophyletic group with the Gyalectales that is characterized by a hemiangiocarpous ascoma ontogeny. The Gyalectales, on the other hand, were included in the Ostropales by Kauff and Lutzoni (2002). Recent results indicate that Agyriales and Pertusariales might be synonymous and that the larger part of the Agyriaceae (*Orceolina, Placopsis, Rimularia, Trapelia* and *Trapeliopsis*) may belong to the Baeomycetales (Lumbsch et al. 2007b). Furthermore, the position of many families (e.g. Arctomiaceae, Thelenellaceae) within the subclass remains uncertain.

Agyriales

Until recently, the order Agyriales comprised lichenized as well as unlichenized species (Rambold and Triebel 1990) and was traditionally treated as suborder Agyriineae of the Lecanorales. Lumbsch (1997) suggested excluding the group from this order because it differed in ascoma ontogeny, ascus type and the faint amyloid reaction of the hymenium. The separation of the order from the Lecanorales and close relationships with the Pertusariales and Ostropales were inferred by Lumbsch et al. (2001a). The phylogenetic relationships of genera within the order are still somewhat uncertain. While *Elixia* seems to be more closely related to the Umbilicariales (Lumbsch et al. 2004b), *Schaereria* was placed close to *Ainoa* (Lumbsch et al. 2001b) or the Ostropales (Lumbsch et al. 2007b). The genus *Ainoa* was described to accomodate *Trapelia geochroa* and *T. mooreana*, after Lumbsch et al. (2001b) found that *Trapelia* was polyphyletic. It was later shown to be closely related to *Baeomyces* (Wedin et al. 2005a; Lumbsch et al. 2007b). The analysis by Lumbsch et al. (2007b) showed *Agyrium* as sister group to Pertusariaceae and Coccotremataceae, referring the bulk of the former Agyriaceae to the Trapeliaceae. The *Trapelia*-type ascus has thus evolved independently in two different lineages of the Ostropomycetidae. The separation of *Agyrium* from the Trapeliaceae is supported by its annulate exciple and richly branched paraphyses (most Trapeliaceae have a cupulate exciple and unbranched paraphyses). Excipular anatomy is also associated with phylogenetic position in the genera *Placopsis* and *Orceolina*. *Placopsis,* sect. *Aspiciliopsis* and *Orceolina*, both with a strongly reduced amphithecium, form a monophyletic sister group to the rest of *Placopsis* (Schmitt et al. 2003a). The immersed position of ascomata, typical for *Orceolina* and the section *Aspiciliopsis*, however, seems to be homoplasious. The different assignments of genera and families within the Agyriales make reconstructions of character evolution within the order (e.g. Lumbsch et al. 2001b) uncertain at present and may be caused by the still rather poor taxon sampling that is mostly non-overlapping between analyses.

Ostropales

As a result of molecular studies, the originally relatively small order Ostropales (Winka et al. 1998) has recently grown considerably and presently comprises the families Coenogoniaceae, Gomphillaceae, Graphidaceae, Gyalectaceae, Phlyctidaceae, Porinaceae, Stictidaceae and Thelotremataceae (Kauff and Lutzoni 2002; Grube et al. 2004a; Lücking et al. 2004; Lumbsch et al. 2004b).

The Gomphillaceae were usually separated from the Asterothyriaceae because of their branched and anastomosing paraphyses and the formation of hyphophores. Lücking et al. (2004) showed that his distinction cannot be held up and that the Gomphillaceae s. lat. are closely related to the Thelotremataceae. The generic delimitations within the Gomphillaceae have been studied by Lücking et al. (2005) based on morphological data, but so far, molecular data are lacking to test their conclusions.

The variability of ascoma types within the Ostropomycetidae is demonstrated by the inclusion of the pyrenocarpous Porinaceae within this subclass. Grube et al. (2004a) pointed out that the perithecia in this family share a special hemiangiocarpous ontogeny that is also found in apothecial groups of Ostropomycetidae and concluded that the perithecia were in fact neotenic apothecia.

The family Stictidaceae in its original circumscription was characterized by thin-walled, narrow asci, needle-like spores and an ascoma margin inspersed with crystals. Molecular studies have confirmed Gilenstam's (1969) conclusion that the family contained lichenized as well as unlichenized species. The lichenized genus *Conotrema* and the non-lichenized *Stictis* are not only congeneric (Winka et al. 1998) but lichenization within this complex is optional and three species of *Stictis* were shown to be each conspecific with a *Conotrema* species (Wedin et al. 2004). *Carestiella* and *Schizoxylon* were found nested within *Stictis*, which shows that the generic delimitations within the family need to be reconsidered (Wedin et al. 2005b).

At present, little is known about the phylogenetic relationships of the Graphidaceae and the generic delimitations of the more than 1,000 mostly tropical species. Close affinities with the Thelotremataceae were assumed and all recent studies demonstrated that the separation of Graphidaceae with lirelliform and Thelotremataceae with rounded ascomata are not supported by molecular data (Grube et al. 2004a; Miadlikowska et al. 2006; Staiger et al. 2006; Mangold et al. 2008b). The morphological and anatomical variability of ascoma and ascospore shapes and anatomies within the resulting Graphidaceae s. lat. is enormous. Traditionally, ascospore septation and colour, structure and pigmentation of the exciple and the presence or absence of lateral paraphyses served to segregate genera, but molecular studies indicate that large morphologically defined genera, such as *Myriotrema, Ocellularia and Thelotrema*, are poly- or paraphyletic (Frisch et al. 2006). However, *Diploschistes, Glyphis, Diorygma, Phaeographis* and *Platygramme* are monophyletic in all analyses (Lumbsch et al. 2004a; Kalb et al. 2004; Frisch et al. 2006; Staiger et al. 2006; Mangold et al. 2008b). The arrangement of ascomata, spore septation, number of spores per ascus and columella structure are homoplasious (Frisch et al. 2006; Staiger et al. 2006). The majority of *Thelotrema* species group together and, within the group, six well-supported clades were found to differ in morphological and

chemical characters, such as ascospore pigmentation (Lumbsch et al. 2008b). The recently segregated genus *Chapsa* appears to be paraphyletic at the base of this group (Lumbsch et al. 2008c; Mangold et al. 2008a, 2008b), but its monophyly within a paraphyletic *Thelotrema* was strongly supported in another analysis (Frisch et al. 2006). The monophyly of *Diploschistes* is strongly supported (Martín et al. 2003), but the hypothesis that *D. ocellatus* with *Lecanora*-like apothecia is a derived member of the *D. scruposus* group (Lumbsch and Tehler 1998) has not been confirmed. Instead, it appeared basal to the rest of *Diploschistes* (Martín et al. 2000, 2003). Mangold et al. (2008a) segregated *Melanotopelia* with a dark pigmented proper exciple and thin-walled, non-amyloid ascospores from *Topeliopsis* with a hyaline exciple and thick-walled, amyloid ascospores. The transfer of the prototunicate genus *Nadvornikia* to the Thelotremataceae (Lumbsch et al. 2004a) demonstrates that ascus types in this family are at least as variable as in other families of Lecanoromycetes. It is at present difficult to assess the systematic value of morphological characters within this family but the studies by Frisch et al. (2006) and Mangold et al. (2008a) indicate that some monophyletic taxa can be circumscribed by morphological and chemical data.

Pertusariales

The circumscription of the Pertusariales has also changed considerably during recent years. As a result of molecular studies, the order is now divided into the four families Coccotremataceae, Megasporaceae, Ochrolechiaceae and Pertusariaceae (Schmitt et al. 2006).

The phylogenetic position of the Coccotremataceae within the Pertusariales was confirmed by Lumbsch et al. (2001c), who also demonstrated that the ascomata of *Coccotrema* are apothecia, and not perithecia as often believed. The placement is supported by the thick-walled, hyaline ascospores and the presence of β-orcinol depsides. The position of Pertusariaceae and Coccotremataceae as sister groups on the same clade was confirmed by Miadlikowska and Lutzoni (2004) and Lumbsch and Schmitt (2002), while the separation of *Lepolichen* and *Coccotrema*, based on thallus morphology, has not been supported by molecular data (Schmitt et al. 2001).

A close relationship between *Megaspora* and *Aspicilia* has been inferred in several recent studies (Ivanova and Hafellner 2002; Schmitt et al. 2006). In the analysis by Ivanova and Hafellner (2002), the Megasporaceae appear in an odd position close to *Lecidea lapicida* and *Bellemerea alpina,* with Pertusariaceae and Ochrolechiaceae as part of the ingroup, but *Anzina* and *Placopsis* as outgroup. In more recent analyses, a relationship with the Ochrolechiaceae was inferred (Miadlikowska et al. 2006; Schmitt et al. 2006; Lumbsch et al. 2007b).

Most species of the Pertusariaceae belonged to the large genera *Pertusaria* and *Ochrolechia*. Based on morphological and chemical data, the distinction of these two genera has always been difficult and many species were shifted around between them. A number of smaller genera and subgenera were distinguished based on morphological (e.g. *Varicellaria* with septate spores, *Melanaria* with brown spores)

or chemical characters (e.g. *Pertusaria* subg. *Monomurata, Pionospora* and *Pertusaria* characterized by the content of chlorinated xanthones, depsides and depsones, respectively). Molecular studies confirmed that *Pertusaria* s. lat. is polyphyletic and comprises most of the segregated smaller genera as well as *Ochrolechia* (Lumbsch and Schmitt 2001; Schmitt et al. 2003b; Schmitt and Lumbsch 2004). *Thamnochrolechia* was found to belong in the Agyriales (Schmitt et al. 2003b). It appears that important characters, such as number of spore wall layers and ascus type, are homoplasious across the Pertusariaceae (Lumbsch and Schmitt 2001). Nevertheless, the major clades within Pertusariaceae are well characterized by secondary metabolites, the amyloid reaction of the ascus wall and number of spores per ascus. Despite the relatively clear distribution of these characters across the tree, the reconstruction of character evolution proved to be difficult for morphological traits. Only few ancestral character states, such as the presence of lecanoric acid in the ancestor of the *Varicellaria*-group, received high posterior probabilities (Lumbsch et al. 2006). The occurrence of septate ascospores in *Varicellaria* and *Loxosporopsis* proved to be of no systematic value. Species of *Pertusaria* with one-celled spores clustered with *V. rhodocarpa* as a sister group to *Ochrolechia* (Schmitt and Lumbsch 2004) and *Loxosporopsis* is part of the Pertusariaceae s. str. (Schmitt and Lumbsch 2004; Schmitt et al. 2006).

Baeomycetales

Based on similarities in ascus structure, the Baeomycetaceae and Icmadophilaceae were, for some time, thought to be lichenized representatives of the Helotiales (Rambold et al. 1993). Molecular data support that both families are not closely related and belong to the Lecanoromycetes (Platt and Spatafora 1999, 2000). A separation of *Dibaeis* from *Baeomyces* and a close relationship with *Icmadophila* was postulated by Gierl and Kalb (1993) based on apothecial colour. This has also been confirmed by molecular data (Stenroos and DePriest 1998; Platt and Spatafora 2000). More recent analyses placed the Icmadophilaceae, including the obligately sterile genera *Siphula* and *Thamnolia*, close to the Pertusariaceae (Kauff and Lutzoni 2002; Miadlikowska and Lutzoni 2004), while the Baeomycetaceae are more closely related to the Ostropales (Stenroos and DePriest 1998; Kauff and Lutzoni 2002). Platt and Spatafora (2000) concluded that the separation of Baeomycetaceae and Icmadophilaceae was supported by secondary chemistry (depsidones in Baeomycetaceae, depsides in Icmadophilaceae). The placement of the chemically variable genus *Siphula* in Icmadophilaceae seemed to contradict this statement. However, *Siphula* is not monophyletic (Stenroos et al. 2002a) and the *S. complanata* and *S. fragilis* groups with dibenzofurans and depsidones, respectively, were transferred to *Parasiphula* in the Coccocarpiaceae (Grube and Kantvilas 2006). Incongruence between chemical and molecular data, despite a lack of recombination, was recently detected in *Thamnolia vermicularis* (Nelsen and Gargas 2009).

Ostropomycetidae: Families with Uncertain Relationships

Wedin et al. (2005a) found a sister group relationship between the enigmatic *Moelleropsis humida* and *Arctomia*. Lumbsch et al. (2005b) described the new genus *Gregorella* to accomodate the species and found that it was placed in the small family Arctomiaceae (but see Högnabba et al. 2009). This family is characterized by cyanobacterial photobionts and shares the same ascus type with the Agyriaceae.

In the analysis by Miadlikowska et al. (2006), the Loxosporaceae (Sarrameanaceae) occupy a basal position in the Ostropomycetidae, but Lumbsch et al. (2007c, 2008c) could not resolve their position with regard to the Lecanoromycetidea and Ostropomycetidae. The genus *Loxospora* appears to be monophyletic and, after the inclusion of *L. lecanoriformis*, morphologically very variable (Lumbsch et al. 2007c).

4.4.4 Lecanoromycetidae

Lecanorales

The Parmeliaceae are the largest and best-studied family of the Lecanoromycetes. Its monophyly – including the Anziaceae, Alectoriaceae, Hypogymniaceae, Usneaceae and the genus *Menegazzia* – has been confirmed in several studies (Mattsson and Wedin 1998, 1999; Wedin et al. 1999; Persoh and Rambold 2002; Arup et al. 2007; Crespo et al. 2007). The family is almost exclusively comprised of foliose and fruticose species, but is probably derived from crustose ancestors. The crustose genera *Gypsoplaca* or *Protoparmelia* were recently inferred as its closest relatives, of which *Protoparmelia* shares the typical cupular exciple with the Parmeliaceae (Arup et al. 2007; Crespo et al. 2007); the crustose *Lecidea rubrocastanea* appears to belong here too (Spribille and Printzen 2007). Related families are the Lecanoraceae, Cladoniaceae and Stereocaulaceae (Wedin et al. 1999; Arup et al. 2007).

During the past 50 years, numerous new genera were segregated mainly from the large genera *Parmelia* and *Cetraria*. Important characters for the delimitation of genera within foliose Parmeliaceae include growth form, cortex anatomy, cell wall polysaccharides, the position of ascocarps and pycnidia and ascospore pigmentation, while the cetrarioid genera were often characterized by ascus anatomy and conidial characters. Because many of these new taxa were not readily accepted and discussions prevailed concerning their rank and monophyly, their circumscription and phylogenetic relationships have been studied in exceptional detail with molecular methods. Crespo et al. (2001) established the monophyly of parmelioid lichens (*Parmelia* s. lat.), but apart from a clade comprising *Cetraria* and *Vulpicida*, the rest of the phylogeny was poorly resolved. Thell et al. (2004) distinguished three well-supported groups of genera with distinct distributional preferences: the predominantly northern cetrarioid species, the mostly tropical *Parmotrema* group incl. *Concamerella, Flavoparmelia,* and *Rimelia*, and the basically southern hemispheric

Xanthoparmelia group incl. *Almbornia, Chondropsis, Karoowia, Namakwa* and *Neofuscelia*. Finally, Crespo et al. (2007) inferred six clades – the parmelioid, alectoroid, psiloparmelioid, cetrarioid, hypogymnioid, and lethariod group. These clades were distinguished by combinations of mostly vegetative characters, such as thallus growth form, cortex structures, position of apothecia and conidia, cell wall glucans and secondary chemistry, but their phylogenetic relationships were largely unresolved.

Of these clades, the strongly supported parmelioid group (Blanco et al. 2004a; Crespo et al. 2007) has been most intensely studied. Blanco et al. (2006) distinguished seven well-supported major clades (the *Xanthoparmelia-, Parmotrema-, Melanelixia-, Parmelia-, Melanohalea-*, *Hypotrachyna-* and *Parmelina*-clade) and mapped the evolution of pseudocyphellae, epicortical pores, usnic acid and atranorin on the phylogeny. The clades are well characterized by combinations of morphological and chemical characters, but their phylogenetic relationships remain uncertain. The monophyly of parmelioid lichens with *Xanthoparmelia*-type lichenan was also confirmed by Blanco et al. (2004b). The genera within the group were all polyphyletic and are now synonymized with *Xanthoparmelia* (Thell et al. 2006; Blanco et al. 2004b). Likewise, Blanco et al. (2005) synonymized several genera with *Parmotrema*, all characterized by an upper cortex of palisade plectenchyma, a pored epicortex, the lack of pseudocyphellae and, interestingly, also by a common lichenicolous fungus, *Abrothallus microspermus*. The brown parmelioid species of *Melanelia* were studied by Blanco et al. (2004a), who found the species to belong to three different clades: *Melanelixia* characterized by a pored epicortex, the lack of pseudocyphellae and the production of lecanoric acid; *Melanohalea* with pseudocyphellae, the lack of epicortical pores and a medulla containing depsidones or lacking secondary compounds; and *Melanelia* s. str. with bifusiform conidia, which falls outside the parmelioid clade. These results largely confirmed a previous study by Guzow-Krzemińska and Węgrzyn (2003) based on a smaller taxon sampling. Another polyphyletic genus, *Hypotrachyna*, was found to fall into two major clades with *Bulbothrix, Cetrariastrum, Everniastrum, Parmelinella* and *Parmelinopsis* intermixed (Divakar et al. 2006). The morphological characters that were traditionally used to segregate these genera were highly homoplasious among clades and no character was found to circumscribe the *Hypotrachyna*-clade. Lumbsch et al. (2008a) found that the two clades of the tropical genus *Hypotrachyna* display significantly higher mutational rates than parmelioid lichens from semi-arid regions. This association between cladogenesis and shifts in environmental conditions suggests adaptive radiation within the Parmeliaceae.

The relatively few species of cetrarioid Parmeliaceae have been split into more than 20 genera, mostly based on differences in ascus and conidial characters and secondary chemistry. Some of these split genera (*Cetrariella, Coelopogon*) received support from molecular analyses (Kärnefelt and Thell 2000; Thell et al. 2002), while others (*Allocetraria, Tuckermanopsis*) proved to be polyphyletic (Thell 1998; Saag et al. 2002; Mattsson and Articus, 2004). The bulk of the cetrarioid lichens form a well-supported group but the parmelioid Cetrariae are excluded (Mattsson and Wedin 1998; Crespo et al. 2001, 2007; Thell et al. 2002).

Two major clades within the cetrarioid lichens are characterized by bifusiform and bacilliform conidia (Thell et al. 2002). Saag et al. (2002), on the other hand, found little correlation between phylogenetic trees based on morphological and molecular data from the cetrarioid genera with globose ascospores. However, this interpretation has to be treated with care, because the "group" is in fact not monophyletic (Thell et al. 2002). A similar problem may affect the study by Mattsson and Articus (2004), who used *Melanelia* as outgroup. This genus was later strongly supported as a member of the cetrarioid clade (Crespo et al. 2007).

In a study on Japanese *Usnea* species, the subgenera *Dolichousnea*, characterized by annular pseudocyphellae, and *Eumitria* with a fistulose axis appeared as strongly supported clades (Ohmura 2002). *Neuropogon*, another infrageneric taxon of *Usnea*, is characterized by a dark pigmentation of cortex and apothecial discs and sometimes treated as a separate genus. Neuropogonoid species of *Usnea* appear monophyletic, but nested within *Usnea* (Articus 2004; Ohmura and Kanda 2004) or paraphyletic within the subgenus *Usnea* when more species are included in the analysis (Wirtz et al. 2006). Analogous to the *Xanthoparmelia* clade discussed above, cortical chemistry, in this case black pigmentation, does not seem to characterize phylogenetic lineages.

In most cases, molecular data seem to support species circumscriptions based on morphological or chemical characters in the Parmeliaceae. Tehler and Källersjö (2001) found that *Parmeliopsis ambigua* and *P. hyperopta* are monophyletic species distinguished by the presence or absence of usnic acid. Within *Parmelia* s. lat. and *Hypogymnia*, well-supported clades were associated with morphological and chemical differences, which led to the description of several new species (Feuerer and Thell 2002; Molina et al. 2004; Divakar et al. 2005b; Argüello et al. 2007; McCune and Schoch 2009). Other studies confirmed the species status of previously synonymized taxa, e.g. *Punctelia jeckeri* (*P. ulophylla*, Crespo et al. 2004b; Thell et al. 2005) or *Parmotrema pseudoreticulatum* (Divakar et al. 2005a). However, there are exceptions. Thell et al. (2000) found that *Cetraria aculeata* was paraphyletic with *C. muricata* embedded. *Punctelia borreri* appears to be paraphyletic as well (Thell et al. 2005), and the distinction of the sexual *Usnea florida* from the asexual, sorediate *U. subfloridana* was also not supported (Articus et al. 2002). Several morphologically circumscribed species of neuropogonoid *Usnea* appeared as para- and polyphyletic on phylogenetic trees (Seymour et al. 2007). Again, care has to be taken when interpreting these results. In a cohesion species approach based on haplotype networks, Wirtz et al. (2008) detected morphological characters that distinguished different lineages of supposedly polyphyletic species in the *U. perpusilla* complex.

The circumscription of the Lecanoraceae has changed considerably in recent years and is still not settled, mostly because genera previously included in the family were found not to belong there. The Lecanoraceae belong to the crown group of Lecanoromycetidae and appear to be most closely related to the clade comprised by Cladoniaceae and Stereocaulaceae (Ekman and Wedin 2000; Miadlikowska et al. 2006). All molecular analyses conducted so far are based on very small taxon sets, which makes it difficult to assess the delimitation of the family. The Lecanoraceae in the broad sense

(including genera, such as *Lecidella, Miriquidica*, *Ramboldia* or *Tephromela*) were mostly reconstructed as para- or polyphyletic (Ekman and Wedin 2000; Miadlikowska et al. 2006; Arup et al. 2007). The genera *Miriquidica, Calvitimela*, *Ramboldia* and *Haematomma* apparently do not belong here (Arup et al. 2007; Lumbsch et al. 2007c). *Lecidella* falls outside the core Lecanoraceae in the analysis by Lumbsch et al. (2008c), but inside in others (Ekman and Wedin 2000; Grube et al. 2004b; Miadlikowska et al. 2006; Kalb et al. 2008). Although *Protoparmelia* was found to be closely related to the Parmeliaceae (see above), a position close to Mycoblastaceae and Lecanoraceae was also inferred (Grube et al. 2004b; Lendemer and Lumbsch 2008).

One of the key characters to circumscribe the Lecanoraceae was the ascus type (Hafellner 1984), but it has become clear that *Lecanora*-type asci are plesiomorphic or polyphyletic and that genera with other ascus types, e.g. *Ramalinora* with a *Biatora*-type ascus, belong to the Lecanoraceae as well (LaGreca and Lumbsch 2001a). Thallus growth form was used to distinguish infrageneric groups within *Lecanora* but neither the umbilicate genus *Rhizoplaca* nor the placodioid *Lecanora* subg. *Placodium* proved to be monophyletic (Arup and Grube 1998, 2000). Secondary chemistry may provide a more reliable character set. *Arctopeltis thuleana* is grouped with the *Lecanora dispersa* group, which often contains xanthones, and *Rhizoplaca* appeared embedded in a species group with usnic acid. The sordidone containing, morphologically very variable species of the *L. rupicola* group form a well-supported clade (Grube et al. 2004b; Blaha and Grube 2007). Species of the group were also characterized by highly similar, orthologous PKS gene sequences (Grube and Blaha 2003).

The Cladoniaceae and Stereocaulaceae are traditionally characterized by a cladoniiform thallus with true podetia (Cladoniaceae) or pseudopodetia (Stereocaulaceae). A number of studies tried to clarify the phylogenetic relationships between "cladoniiform" lichens, also including species from the Icmadophilaceae and Baeomycetaceae (Stenroos and DePriest 1998; Stenroos et al. 2002a). The last two families were shown to be more closely related to the Pertusariales and Gyalectales and are now placed in the Ostropomycetidae (see above). Thallus characters were not consistently used to assign genera to both families and molecular data confirm that podetia or pseudopodetia evolved several times independently, although the re-circumscribed Cladoniaceae and Stereocaulaceae are monophyletic (Stenroos and DePriest 1998; Wedin et al. 2000a; Myllys et al. 2005; Zhou et al. 2006). For example, the genera *Cladia* and *Ramalea* with pseudopodetia have often been classified in the Cladoniaceae and, based on molecular data, *Pilophorus* has been transferred from the Stereocaulaceae to the Cladoniaceae as well (Stenroos and DePriest 1998; Wedin et al. 2000a). *Austropeltis* and *Neophyllis*, on the other hand, have been included in the Sphaerophoraceae (see below). The genus *Cetradonia* with solid podetia was first segregated from the Cladoniaceae (Stenroos and DePriest 1998), but later found to be a sister group to *Gymnoderma*, which differs in ascus structure from the rest of the Cladoniaceae (Zhou et al. 2006). The finding that thallus characters, such as the formation of pseudopodetia, are of little use for the delimitation of the Stereocaulaceae has also been supported by the inclusion of the crustose genera *Muhria* (Myllys et al. 2005), *Lepraria* (Ekman and Tønsberg 2002)

and *Hertelidea* (Printzen and Kantvilas 2004) into the family. The crustose growth form seems to be plesiomorphic within *Stereocaulon* and perhaps within the Stereocaulaceae as a whole. *Muhria* was also believed to differ from *Stereocaulon* in a hemiangiocarpous ascocarp development but this type is in fact also found in *Stereocaulon* (Timdal 2002).

Most species of *Cladonia* and *Stereocaulon* are morphologically very variable and the genera received considerable attention by systematists, resulting in elaborate infrageneric classifications (e.g. Lamb 1977; Ahti 2000). In a study based on molecular, morphological and chemical characters, Stenroos et al. (2002b) demonstrated that *Cladina* was monophyletic and part of *Cladonia*, but did not confirm any of the sections of *Cladonia* distinguished in the most recent classification of the genus (Ahti 2000). It also emerged from this study that most morphological and chemical characters are highly homoplasious within *Cladonia*. On the other hand, the study by Högnabba (2006) revealed a good correlation of monophyletic groups with infrageneric taxa of *Stereocaulon*. Morphological characters seem to be useful for delineating monophyletic groups but not for species or for reconstructing phylogenetic relationships between these groups. Many of the morphologically variable species of *Cladonia* and *Stereocaulon* proved to be polyphyletic (Stenroos et al. 2002b; Myllys et al. 2003; Högnabba 2006). The obligately sterile genus *Lepraria* has gained considerable attention in recent years and numerous new species have been described (Nelsen et al. 2008). Because distinguishing morphological characters are largely lacking, chemical characters have played an important role in the delimitation of species. Several studies have confirmed that chemically deviating leprose species, e.g. *L. obtusatica* and *L. usnica*, do not belong in the genus and that the leprose growth form evolved independently in different families of Lecanorales (Ekman and Tønsberg 2002; Nelsen et al. 2008). The genus *Leproloma* appears polyphyletic within *Lepraria*. Chemical characters seem to be useful for the delimitation of species but do not reflect phylogenetic relationships within *Lepraria* (Ekman and Tønsberg 2002; Fehrer et al. 2008; Nelsen and Gargas 2008). A species level study by Tretiach et al. (2009), however, demonstrated considerable chemical variability within the two genetically and morphologically well-characterized species *L. isidiata* and *L. santosii*.

The fruticose Ramalinaceae and crustose Bacidiaceae were kept separate until Ekman (2001) found that they were not reciprocally monophyletic. Based on differences in ascus structure, spore and exciple anatomy, the Bacidiaceae had a turbulent history being first divided into several families, which were then gradually reunited (e.g. Biatoraceae, Lecaniaceae, Phyllopsoraceae) or shown to be phylogenetically unrelated (e.g. Tephromelataceae, Squamarinaceae). Some studies inferred a close relationship between Ramalinaceae and Sphaerophoraceae (Ekman and Wedin 2000; Persoh et al. 2004; Miadlikowska et al. 2006), whereas others indicated relationships with the Micareaceae (Andersen and Ekman 2004). The broadest taxon sampling so far placed the Psoraceae between Ramalinaceae and a separate family Bacidiaceae and revealed the Micareaceae and Pilocarpaceae as the closest relatives to this clade (Andersen and Ekman 2005). Finally, in the analysis

by Ekman et al. (2008), Ramalinaceae and Bacidiaceae are again united on the same branch. It seems that phylogenetic reconstructions are highly influenced by taxon sampling, which is still rather poor. For example, only one species of the large genus *Phyllopsora* has so far been included in a molecular study (Printzen and Lumbsch 2000). The polyphyletic Micareaceae were united with the Pilocarpaceae by Andersen and Ekman (2005). Almost all traditionally circumscribed genera within these families are polyphyletic and generic delimitations are in need of revision (Ekman 2001; Andersen and Ekman 2005; Reese Næsborg et al. 2007). In some cases, hymenial pigmentation seems to indicate phylogenetic relationships (Ekman 2001; Printzen et al. 2001). Ascus characters, however, do not seem to reflect relationships in the Ramalinaceae s. lat. and Pilocarpaceae. The typical Ramalinaceae ascus apex (the *Bacidia*- or *Biatora*-type) can be found in other families (e.g. Lecanoraceae) as well. Moreover, ascus types that were previously used to refer *Micarea* to the suborder Cladoniineae and *Bacidia* to the Lecanorineae (Rambold and Triebel 1992), occur in both families. The *Lecanora*-type ascus was inferred to be ancestral in the group (Andersen and Ekman 2005; Ekman et al. 2008).

Muggia et al. (2008a) studied the species complex around *Tephromela atra* in the Mycoblastaceae and found a relatively clear association between ecologically different morphotypes and clades on an ITS-tree. However, the pattern got more obscure when data from β-tubulin and PKS genes were included.

Due to their prototunicate asci, the Sphaerophoraceae were traditionally classified close to the Caliciales. Wedin et al. (1998) showed that prototunicate asci had evolved independently in the Mycocaliciaceae and that the Sphaerophoraceae were nested within the Lecanorales, a position also supported by pycnidial ontogeny and conidiophores. The order Caliciales has since been demonstrated to be polyphyletic and the Caliciaceae are treated as a synonym of the Physciaceae. This highlights the importance of taxon sampling not only for the analysis but also for the interpretation of molecular results. The data set by Wedin et al. (1998) did not include sequences from the Caliciaceae, so that the hypotheses that Sphaerophoraceae are part of the Lecanorales or part of the Caliciales were not contrastive. Wedin and Döring (1999) transferred the genera *Austropeltis* and *Neophyllis* from the Cladoniaceae to the Sphaerophoraceae. This was not only supported by molecular data but also by the discovery that the boundary tissue thought to be typical for *Austropeltis* and *Neophyllis* was also present in *Sphaerophorus* (Döring et al. 1999). Wedin et al. (2000a) found close relationships between this family and the Ramalinaceae (Bacidiaceae). Molecular data support the distinction of two *Sphaerophorus* species with different thallus growth (Högnabba and Wedin 2003).

Teloschistales

Similarities in ascospore morphology and ontogeny (thickened pigmented spore-walls that are often ornamented) caused Wedin and Tibell (1997) to suggest a close relationship between Physciaceae and Caliciaceae. Wedin et al. (2000b) confirmed

this hypothesis and both families are now treated as synonyms. Other similarities, such as the frequent occurrence of stalked ascomata or melanized excipula, also occur in several other groups of lichens and can probably not be regarded as synapomorphies. The phylogenetic analysis by Lohtander et al. (2000) included only few crustose members of the family, but confirmed an earlier hypothesis by Rambold et al. (1994) that species with *Bacidia*- and *Lecanora*-type asci belong to two different evolutionary groups within the family. Crustose and foliose species were found in both groups – the *Buellia*-group and the *Physcia*-group. Two later studies confirmed the monophyly of both species groups (Grube and Arup 2001; Wedin et al. 2002). Wedin et al. (2002) also found that calicioid members of the family with prototunicate asci belong into the *Buellia*-group. Helms et al. (2003) mapped morphological and chemical characters on an extended phylogeny of Physciaceae and showed that ascus type and hypothecial pigmentation were positively correlated with monophyletic groups and made good systematic characters, while growth form, ascospore types, exciple structure, and the presence or absence of atranorin were highly homoplasious. These molecular results were in conflict with those of previous cladistic analysis based on morphological and chemical data, which suggested that foliose and crustose members of the Physciaceae belonged to different clades (Nordin and Mattsson 2001). Therefore, it may be difficult, if not impossible, to reconstruct the evolutionary history of lichens based on traditional characters.

Several studies have investigated the phylogenetic relationships of genera within the Physciaceae. Molina et al. (2002) synonymized *Diploicia* with *Diplotomma* based on an ITS phylogeny, although both formed well-supported monophyletic clades. Within *Physconia,* molecular data largely confirmed previous hypotheses about sub-generic relationships and the importance of morphological characters for distinguishing species groups. Cubero et al. (2004) found that the colour of the lower thallus surface and the morphology of rhizines are good characters to separate series within *Physconia*. The upper cortex is useful for distinguishing species, but seems of little use for the distinction of series or species groups (Cubero et al. 2004; Divakar et al. 2007). Likewise, Lücking et al. (2008a) confirmed that a combination of chemistry, lobe colour and reproductive mode distinguishes monophyletic groups within the *Heterodermia obscurata* group. Tibell (2001) found that the catenulate asci of *Cybebe gracilenta* are autapomorphic and that the species in fact belongs to *Chaenotheca*. Another study revealed that the genera *Calicium* and *Cyphelium*, as traditionally circumscribed, are polyphyletic with *C. adaequatum* closely related to *Tholurna* (Tibell 2003). These two species share an campanulate capitulum and ascospores with a strong surface ornamentation. However, the phylogenetic relationships within the Caliciaceae remained unclear because the backbone of the phylogeny was largely unresolved.

The family Teloschistaceae is, among others, well characterized by the production of anthraquinones. Within the family, thallus characters have traditionally played a major role in delimiting genera and subgeneric taxa. However, the borders between these traditional genera have always been somewhat fuzzy, especially between the lobate species of *Caloplaca* sect. *Gasparrinia* and the foliose genus

Xanthoria. Recent molecular investigations confirmed that the largest genera of the Teloschistaceae, *Caloplaca, Fulgensia, Teloschistes* and *Xanthoria* are polyphyletic. Kasalicky et al. (2000) showed that *Fulgensia australis* and *F. schistidii* have to be removed from the genus, but that the bulk of *Fulgensia* species is monophyletic. These results were confirmed by Gaya et al. (2003, 2008), who also showed that almost all traditional subgeneric taxa within *Caloplaca* are polyphyletic. However, smaller previously recognized groups, such as the *Caloplaca saxicola* group or the *C. aurantia* group, seem to be monophyletic. Søchting et al. (2002) found the *Xanthoria ulophyllodes* group (incl. *Xanthomendoza*) to be monophyletic and well characterized by bacilliform conidia and true rhizines and recombined the species of this group into *Xanthomendoza*. Only few major phylogenetic lineages are supported by morphological and/or chemical characters. According to Søchting and Lutzoni (2003), a clade comprising most *Xanthoria* species and the former *C. saxicola* group is characterized by more or less of ellipsoid conidia, a paraplectenchymatous upper cortex, and parietin as major secondary metabolite, while *Fulgensia* and a few phylogenetically related species contain fragilin. Gaya et al. (2008) suggested using the name *Seirophora* for a group of former *Teloschistes* spp. without anthraquinones in the thallus. Because the taxon sampling is still rather poor, it seems at present unclear whether chemical characters really support certain phylogenetic lineages within the Teloschistaceae. For example, in the analysis by Arup and Grube (1999), *Caloplaca demissa, C. variabilis* and another species without anthraquinones formed a monophyletic group that was not recovered in the analysis of Gaya et al. (2008). Based on an extended sample, Muggia et al. (2008d) found *Caloplaca* subgenus *Pyrenodesmia* to be monophyletic and *C. demissa* closely related, but anthraquinone-containing species intermixed on the same clade. At the species level, *C. citrina* was found to comprise five (Arup 2006) and *C. holocarpa* has four morphologically distinct species (Arup 2009). Lindblom and Søchting (2008) demonstrated that the morphologically similar *Xanthomendoza borealis* and *Xanthoria candelaria* are genetically and anatomically different species, while Vondrák et al. (2008) confirmed the position of two new *Caloplaca* species in the *C. cerina* group, a placement that was also supported by chemical data.

Peltigerales

Species of the Peltigerales may form symbioses with either cyanobacteria alone (bipartite species) or green algae and cyanobacteria (tripartite species). Associations with only green-algal species are rare. The systematic relationships within the group have always been uncertain, resulting in an unusually high number of genera placed as “incertae sedis”. The monophyly of the Peltigerales has been confirmed (Wiklund and Wedin 2003; Miadlikowska and Lutzoni 2004). Two main clades can be distinguished within the order: the Collematineae, comprising the Collemataceae, Pannariaceae and Placynthiaceae; and the Peltigerineae with the mostly large foliose Peltigeraceae, Nephromataceae, Massalongiaceae and

Lobariaceae (Miadlikowska and Lutzoni 2004; Wedin and Wiklund 2004; Wedin et al. 2007). The Lobariaceae (including *Sticta, Lobaria* and *Pseudocyphellaria*) were reconstructed as monophyletic, while the Pannariaceae, *Solorina, Pseudocyphellaria, Lobaria, Leptogium* and *Degelia* appeared poly- or paraphyletic (Thomas et al. 2002; Miadlikowska and Lutzoni 2004). *Lobaria* was also reconstructed as monophyletic by Wiklund and Wedin (2003) and Wedin and Wiklund (2004), who attributed this deviating result to differences in taxon sampling.

The distinction of genera within the Lobariaceae was traditionally based on thallus characters, such as presence and absence of pseudocyphellae and cyphellae, and seemed more or less straightforward. Molecular data, however, indicate that groups based on thallus characters are not monophyletic and, hence, that these characters do not indicate phylogenetic relationships. The morphologically circumscribed genera *Lobaria* and *Pseudocyphellaria* appear highly polyphyletic in several recent analyses (Thomas et al. 2002; Stenroos et al. 2003). Interestingly, the pigmentation of the medulla correlates with (poorly supported) clades of *Pseudocyphellaria* (Thomas et al. 2002) and characters of the cyphellae, isidia and thallus lobes or chemistry agree well with phylogenetic species in *Sticta* (McDonald et al. 2003). Tripartite species of Lobariaceae form morphologically extremely deviating thalli, so-called photosymbiodemes, with either cyanobacteria or green algae (James and Henssen 1976; Hawksworth 1988). The identity of cyanobacterial with green-algal photomorphs has repeatedly been confirmed by molecular methods and growth experiments (Goffinet and Goward 1998; Thomas et al. 2002; Stenroos et al. 2003).

Miadlikowska and Lutzoni (2000) investigated the infrageneric phylogeny of *Peltigera* (Peltigeraceae) and found eight well-supported monophyletic groups that showed some overlap with the infrageneric groups suggested by Holtan-Hartwig (1993) and were characterized by morphological and chemical differences, especially differences in terpenoid composition. This is also true for the seven monophyletic subgroups within the section *Peltigera*, found by Miadlikowska et al. (2003). Fifteen of seventeen accepted species within the group could be clearly circumscribed by morphological characters. Just as in Pannariaceae and *Nephroma*, *Peltigera* species associated with identical photobionts did not form monophyletic groups.

The genus *Nephroma* (Nephromataceae) was studied by Lohtander et al. (2002), who found it to be monophyletic and sister to *Peltigera*. *Nephroma expallidum* and *N. arcticum* with green-algal and cyanobacterial photobionts occurred in different clades among purely cyanobacterial *Nephroma* species, which shows that a switch to green-algal photobionts occurred several times independently. Just as in *Peltigera*, chemical characters seem to be correlated with certain clades, e.g. the anthraquinone-containing species *N. laevigatum* and *N. tangeriense* form a clade in Lohtander et al. (2002). However, Piercey-Normoore et al. (2006) found that *N. expallidum* with only terpenoids belongs to the same group.

Due to the transfer or splitting of genera, the relatively small family Pannariaceae has experienced almost constant changes since the 1970s. Most species traditionally included in the family are associated with cyanobacteria, but *Psoroma*

and *Psoromidium* have green-algal photobionts. In addition to photobiont choice, ascus anatomy has played a role in defining taxa within Pannariaceae. The family and most of its genera have been shown to be polyphyletic and different photobionts may occur in one and the same genus (Ekman and Jørgensen 2002). *Psoroma* proved to be highly polyphyletic (Passo et al. 2008). It seems that ascus characters may be more reliable indicators of phylogenetic relationships within this family. *Fuscopannaria* subg. *Micropannaria* shows affinities to the Lobariaceae (Ekman and Jørgensen 2002) and *Moelleropsis humida* was transferred to the new genus *Gregorella* in the Arctomiaceae (Lumbsch et al. 2005b). The position of *Degelia* seems equivocal at present. Close affinities with *Peltigera* were inferred by Thomas et al. (2002), while Wedin et al. (2007) found it belongs to the Pannariaceae.

The genera *Massalongia, Polychidium* and *Leptochidium* were placed in a separate family Massalongiaceae in the Peltigerineae by Wedin et al. (2007). A similar ascus structure and the hemiangiocarpic apothecial ontogeny of all three genera support their close relationship. A possible close relationship to *Nephroma* as postulated by Miadlikowska and Lutzoni (2000) remains unresolved.

Few studies have dealt with the Collemataceae. Passo et al. (2008) found that *Leptogium* was more closely related to the Pannariaceae than the Lobariaceae. Molecular data supports the distinction of three morphologically well-defined species in the *L. lichenoides* group (Otálora et al. 2008).

Families with Uncertain Relationships

The Lecideaceae was the largest family within Zahlbruckner's (1926) system and has long been recognized as an artificial assemblage of similar species. Since Hafellner's (1984) pioneer study, ascus types have been used to segregate the family into phylogenetic lineages and members of the Lecideaceae have been distributed over dozens of families. *Lecidea* was found to be polyphyletic and embedded in a paraphyletic genus *Porpidia* (Buschbom and Mueller 2004; Buschbom and Barker 2006). Species with a *Porpidia*-type ascus were distributed over several families. These results have been confirmed by Andersen and Ekman (2005). *Lecidea tessellata*, the only member of subgenus *Cladopycnidium*, is only distantly related to the rest of *Lecidea*.

The systematic position of Rhizocarpaceae is still unresolved. Together with the Catillariaceae they appear outside the Lecanoromycetidae. Miadlikowska et al. (2006) tentatively placed them under the name Rhizocarpomycetidae together with the Catillariaceae. The genera *Catolechia* and *Poeltinula* differ from *Rhizocarpon* mainly in spore characters but do not seem to be separable from *Rhizocarpon* based on molecular data (Ihlen and Ekman 2002). Spore septation, presence or absence of rhizocarpic acid, or amyloid reaction of the medulla were traditionally used to subdivide *Rhizocarpon* into subgenera, but all of these characters were reconstructed as highly homoplasious (Ihlen and Ekman 2002).

4.5 *Lichinomycetes*

The moderately sized family Lichinaceae is characterized by cyanobacterial photobionts and the presence of prototunicate asci in most species. The family was long regarded as a basal lineage of ascomycetes, together with the prototunicate Caliciaceae and Mycocaliciaceae. It is now firmly established that these families are unrelated and that prototunicate asci have evolved multiple times by reduction. The Lichinaceae cluster outside the Lecanoromycetes in all analyses (Lutzoni et al. 2001; Schultz et al. 2001; Miadlikowska and Lutzoni 2004; Bhattacharya et al. 2005; Miadlikowska et al. 2006; Högnabba et al. 2009) and seem to be related to *Thelocarpon* and *Biatorella* (Bhattacharya et al. 2005; Reeb et al. 2004). A position outside the Lecanoromycetes is also supported by polysaccharides that closely resemble those of some Eurotiomycetes (Prieto et al. 2008). Reeb et al. (2004) described the new class Lichinomycetes to accomodate them. The sister group relationships of the Lichinomycetes are still unsolved. They appear as sister group to the Lecanoromycetes (Miadlikowska and Lutzoni 2004; Miadlikowska et al. 2006), Arthoniomycetes, Eurotiomycetes and Lecanoromycetes (Schultz et al. 2001), Arthoniomycetes and Dothideomycetes (Reeb et al. 2004), or within an unresolved clade composed of Dothideomycetes, Eurotiomycetes, Sordariomycetes, Leotiomycetes and the Acarosporomycetidae and Candelariomycetidae (Lücking et al. 2008b). Schultz et al. (2001) confirmed the monophyly of Lichinales, including Lichinaceae and Peltulaceae. *Peltula* was, however, paraphyletic within the Lichinaceae in the analysis of Högnabba et al. (2009). The genus *Heppia* proved to be embedded within the Lichinaceae and the Heppiaceae were reduced to synonymy (Schultz et al. 2001; Schultz and Büdel 2003). The placement of *Heppia* within the Lichinaceae and the separation of the Peltulaceae are supported by ascus characters. As far as can be judged from the limited data available, the generic delimitations within the Lichinaceae are well supported by molecular data. So far, only *Peccania* has been shown to be paraphyletic (Schultz and Büdel 2003). However, traditional species delimitations within *Peltula* are in surprising conflict with phylogenetic results (Rauhut 2006). Lücking et al. (2008b) found that *Eremithallus costaricensis*, a species with a *Trentepohlia* photobiont that is morphologically similar to Ostropales, constitutes a new lineage of Lichinomycetes sister to the Lichinales and described the new order Eremothallales.

5 Systematics of Lichenized Basidiomycota

Although few lichens belong to the Basidiomycota, some of the first molecular studies on fungi already included representatives of this group (Gargas et al. 1995). However, because they constitute less than one percent of all known Basidiomycota, lichenized species have played no role in resolving higher-level phylogenies of this division and were rarely included in higher-level phylogenetic studies of

Basidiomycota (Lutzoni et al. 2004; Matheny et al. 2006). A recent study including eleven obligately and two facultatively lichenized species of Basidiomycetes (Lawrey et al. 2007) revealed that these species belong to different orders of the Homobasidiomycetes. *Multiclavula* appears monophyletic within the Cantharellales, *Marchandiomphalina foliacea* is a member of the Corticiales, *Dictyonema* and *Lichenomphalia* belong in the Agaricales, and the facultatively lichenized *Schizophora paradoxa* and *Resinicium bicolor* are members of the Hymenochaetales. Whether or not morphological characters support molecular results has rarely been discussed or tested (Lutzoni and Vilgalys 1995b; Redhead et al. 2002).

The genus *Omphalina* has received more attention among the lichenized basidiomycetes. Early studies demonstrated that *Omphalina* is polyphyletic with different species related to *Hygrocybe, Rickenella* and *Clitocybe* (Lutzoni 1997; Lutzoni and Pagel 1997). In accordance with previous hypotheses (Redhead and Kuyper 1987; Norvell et al. 1994), the five lichenized species *O. ericetorum, O. grisella, O. hudsoniana, O. luteovitellina* and *O. velutina* were found to form a monophyletic group (Lutzoni and Vilgalys 1995a; Lutzoni 1997; Lutzoni and Pagel 1997; but see Moncalvo et al. 2000) as did two species of *Multiclavula* included in these studies. Redhead et al. (2002) introduced the new generic name *Lichenomphalia* for this group, which is also moderately supported by morphological data (Lutzoni and Vilgalys 1995b; Redhead et al. 2002). Lutzoni and Pagel (1997) reported higher substitution rates in mutualistic than in non-mutualistic species of their dataset. The sterile *Omphalina foliacea* was found to belong in the Basidiomycota but its phylogenetic position still seems to be a bit uncertain, being either assigned to the Hymenochaetales (Palice et al. 2005) or the Corticiales (Lawrey et al. 2007; Diederich and Lawrey 2007).

Multiclavula was often used as an outgroup in studies on *Omphalina* (see above). Nelsen et al. (2007) included four species of the genus and confirmed their monophyly, although the new species *M. ichthyiformis* differed by the lack of an amphigenous hymenium, the presence of crystals in the lamina and other characters from typical *Multiclavula*-species. Two recently described species of *Multiclavula* (Fischer et al. 2007) were shown to be closely related to *Lepidostroma*, with which they form an isolated lineage, the Lepidostromataceae (Ertz et al. 2008). This last result probably merits careful reexamination, because it places Basidiomycetes with morphologically indistinguishable fruiting bodies in two widely separated lineages, the Cantharellales and the Atheliales.

6 Conclusion

The view that the systematic value of certain characters is usually restricted to a taxonomic group and cannot simply be transferred to other such groups has been commonplace among taxonomists for a long time. Nevertheless, the classification of ascomycetes has long suffered from a schematic application of character sets, such as types of ascoma ontogeny or ascus types. These characters have not only

been used to circumscribe certain groups but also to postulate phylogenetic relationships between different taxa. Molecular analyses offer the opportunity to critically evaluate the usefulness of morphology and secondary chemistry in lichen systematics. The results so far support the traditional view that there are no universally applicable traits. Key characters within the ascomycetes seem to have evolved independently or changed their character states frequently in the course of evolution. Whether they are systematically "useful" or not needs to be decided from case to case. Molecular data have become indispensable for the delimitation of evolutionary groups because they allow the detection of groups without prior assumptions on the importance of certain characters. At the same time, molecular methods have turned lichen systematics into a hypothesis-driven science that depends on the results of careful morphological, ontogenetical and chemical studies. In this way morphological, chemical and molecular data depend on each other and none can substitute for the other.

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Ecology

Systemic Resistance Induction by Vascular and Airborne Signaling

Martin Heil and Jurriaan Ton

Contents

Abstract Plants cannot escape spatially from harmful abiotic conditions or enemy attack. Therefore, they use both constitutive and inducible defense mechanisms to fend off pathogens and herbivores. Resistance induced in response to local attack is often expressed systemically, i.e., in yet undamaged organs. The search for the

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long-distance signals mediating systemic resistance led to the identification of hormones, such as jasmonic acid (JA), salicylic acid (SA), and ethylene, as well as volatiles and small RNA molecules. This research also revealed that different plant species may use different hormones to mount phenotypically similar resistance responses. Long-distance signals can directly activate defense, or prime for stronger and faster defense induction. Earlier research has focused on vascular transport of signaling metabolites, but volatiles can play a critical role as well. As volatiles move freely through air, they can prime and induce resistance in remote organs of the same individual and in neighboring plants. We compare the advantages and restraints of vascular and airborne signals for the plant and discuss how they can act in synergy to achieve an optimized resistance in distal plant parts.

1 Introduction

Plants are sessile organisms, which cannot escape from a harmful environment. How are plants, then, able to cope with changing conditions? The answer is easy: They change themselves! Phenotypic plasticity affects almost every aspect of plant life: leaf shape, growth rate, where and into which direction to grow new shoots and roots. Plants deploy surprisingly "intelligent" strategies to optimize survival in their constantly changing and often challenging environment. For example, most plants continuously face an ongoing threat of attack by harmful organisms – herbivores and pathogens that exploit plant tissue as a source of energy and nutrients. Defense strategies to combat these threats comprise anatomical features, such as heavily lignified cell walls, trichomes, thorns and spines, and chemical substances that make the tissue less attractive, toxic or simply difficult to digest or infect. Since most of these defense mechanisms are costly, plants have evolved a plethora of inducible defense mechanisms, which are activated only in response to attack.

1.1 Systemic Induced Resistance

Because many pathogens and herbivores are mobile, local infection or infestation has a high predictive value for future attack of the distal, yet undamaged plant parts. To counteract complete destruction, *induced resistance* (IR) often spreads systemically from the damaged tissue to the yet unaffected organs (Karban and Baldwin 1997). In some cases, however, successful localized defense expression may suffice to avoid further disease spreading. Therefore, plants possess an additional strategy to avoid unnecessary investments in systemic defense mechanisms: priming. Primed tissues do not express costly defense mechanisms, but they mount their inducible defense arsenal faster and/or stronger than unprimed ones upon subsequent attack by pathogens or insects (Conrath et al. 2006).

The objective of this chapter is (1) to give a short overview of systemic plant responses to local attack and (2) to present examples of mobile defense signals that mediate these responses. We follow the most widely used definitions and use the term "*systemic acquired resistance*" (SAR) for a pathogen-induced systemically expressed resistance against various pathogens, while "*wound response*" (WR) is used for all resistance phenomena that can be observed after herbivore feeding, although chemical elicitors certainly mediate a plant's response to damage that is inflicted by herbivores. IR is generally used for all plant responses to pathogen infection or herbivore feeding, which reduce damage by the attacking organism.

1.2 Systemic Signaling Mediates Resistance to Pathogens and Herbivores

The first evidence for systemically IR came from Frank Ross in the1960s, who demonstrated that inoculation with tobacco mosaic virus (TMV) on lower leaves of tobacco resulted in enhanced resistance to a second infection in the upper leaves (Ross 1961). This SAR response was first detected at 2 days after primary virus infection and reached a maximum at 7 days, demonstrating that the plant requires time to generate, transport, and deliver a resistance-inducing signal to the upper leaves. Ten years later, Green and Ryan (1972) reported that tomato responds to insect feeding by local and systemic production of proteinase inhibitors (PIs), which are compounds that affect protein digestion by insects. Supplying extracts from wounded leaves to excised, but otherwise undamaged, plants could trigger PI production as well: An observation pointing to the existence of a mobile signal in the WR (Ryan 1974).

Well known examples of systemically expressed plant responses to local attack by pathogens are small secondary molecules, commonly referred to as "phytoalexins," and "pathogenesis related" (PR) proteins (Van Loon 1997; Dangl and Jones 2001). Resistance against insects, in contrast, is mainly obtained by toxic secondary metabolites or PIs and indirectly by attracting "enemies of the plants' enemies": herbivore-induced volatile organic compounds (VOCs) and extrafloral nectar (EFN) signal the presence of herbivore prey to predators and parasitoids, enhancing parasitation and predation pressure on herbivores and thereby functioning as an "*indirect defense*" (Dicke et al. 2003; Heil 2008).

Certain herbivore-induced VOCs can also repel herbivores (De Moraes et al. 2001; Kessler and Baldwin 2001), while other VOCs have antifungal or antimicrobial effects (Peñuelas and Llusiá 2004; Kishimoto et al. 2005; Shiojiri et al. 2006) or protect plants from abiotic stress (Velikova et al. 2005; Behnke et al. 2007). Likewise, phytoalexins can be toxic to animals, and classes of secondary compounds, such as furanocoumarins, are active against bacteria, fungi, viruses, insects, molluscs, and vertrebrates (Berenbaum and Zangerl 1999). Although the separation of SAR and WR is used here for historical reasons, we should be aware that IR is achieved by network-like and in part, multifunctional responses.

2 The Mechanisms Behind Systemic Plant Defenses

2.1 Local Perception of Attack

The early stages of pathogen infection are usually not associated with heavy damage, but involve an intimate contact of plant cells with the attacking microbe. As microbes contain specific compounds that are absent from intact plants, immune responses against microbial pathogens are typically activated upon recognition of small molecular motifs, known as pathogen-associated molecular patterns (PAMPs, also termed MAMPs for microbe-associated molecular patterns) (Jones and Dangl 2006; Schwessinger and Zipfel 2008). While PAMP-triggered immunity protects against a majority of potentially pathogenic microbes, some specialist pathogens have evolved to circumvent or suppress this form of immunity by deploying effector proteins. To counteract this virulence strategy, plants have co-evolved the ability to recognize and respond to these effectors (Jones and Dangl 2006). The resulting effector-triggered immunity (ETI) requires the presence of plant resistance (R) genes and involves a rapid induction of defensive mechanisms that is often associated with a hypersensitive response (HR).

In short, successful responses to pathogen infection usually require a "non self-recognition." Insect feeding, by contrast, inevitably leads to massive cell damage, and mechanical damage only can in some plant species mimic the response to natural herbivore damage (Heil et al. 2001, 2004; Mithöfer et al. 2005; Major and Constabel 2006). This observation is in line with the resistance-eliciting effects of cell wall fragments, like pectines (Doares et al. 1995), oligosaccharins (Creelman and Mullet 1997), and peptides (Narváez-Vásquez et al. 2005). In other plant species, however, insect feeding and wounding cause overlapping but not identical responses (Kessler and Baldwin 2002). Many researchers reported that insect regurgitate needs to be applied to mechanically inflicted wounds to fully mimic the response to insect feeding. This observation led to the introduction of the term "herbivore-associated molecular patterns" (HAMPs) (Felton and Tumlinson 2008), in analogy to PAMPs or MAMPs. Well-known examples of HAMPs are fatty acid–amino acid conjugates (FACs), such as volictin (Alborn et al. 1997), chemically related oxylipins (Koch et al. 1999; Halitschke et al. 2001) and peptides (Pearce and Ryan 2003), such as systemin (Peña-Cortés et al. 2005) and inceptin (Schmelz et al. 2006).

Although physically located in the saliva of insects, most HAMPs are partially or completely derived from plant molecules. For instance, the 18 amino acid-peptide systemin is processed from a larger protein of plant-origin, called prosystemin (McGurl et al. 1992), whereas inceptin is a degradation product of a plant ATPase (Schmelz et al. 2006). Even FACs contain a plant-derived fatty acid portion (Paré et al. 1998). As plant-derived products, most specific WR elicitors originate from taxonomically restricted plant groups and are concurrently only active in plants belonging to these taxonomic groups. For example, volicitin induces defense expression in maize but proved to be inactive in lima bean (Koch et al. 1999), systemin functions only in a small subset of solanaceous species

but even not in another member of the same family, *Solanum nigrum* (Schmidt and Baldwin 2006), and bruchins are active only on certain pea (*Pisum sativum*) genotypes (Doss et al. 2000; Doss 2005). In analogy to ETI, HAMPs could be regarded as the second layer in the plants' counter-evolutionary response to herbivory, while merely wound-derived signals represent the first layer of defense elicitors, triggering a general immune response against which insects have evolved suppressive strategies.

Indeed, herbivores that can suppress the plant's innate WR have been found. For instance, *Manduca sexta* larvae trigger an ethylene burst in *Nicotiana attenuata* to suppress wound-inducible nicotine accumulation (Kahl et al. 2000). Furthermore, activity by the enzyme glucose oxidase from the salivary gland of *Helicoverpa zea* was reported to suppress nicotine accumulation in *Nicotiana tabacum* and to reduce PI activity in tomato (Musser et al. 2002). Hence, both plant–pathogen and plant–herbivore interactions are the result of an evolutionary arms race that involves multiple layers of defense regulation by the host plant and defense deregulation by the attacking organism.

2.2 Cellular Signaling Pathways Mediating Elicitor Perception and Signal Production

The early signaling events preceding the production and translocation of long-distance defense signals are complex. Typically, perception of PAMPs, MAMPs, or HAMPs is initiated by pattern recognition receptors (PRRs) in the plasma membrane or cytosol. Activation of these PRRs is often associated with rapid fluctuations in the plasma membrane potential, cytosolic calcium concentrations (Maffei et al. 2007b), and even endocytosis of plasma membrane-localized PRRs (Robatzek 2007). These events activate downstream signaling pathways in which protein phosphorylation cascades play a central role (Peck 2003). The cross-talk between these pathways will result in activation of specific sets of defense-related transcription factors (Asai et al. 2002; Skibbe et al. 2008), which ultimately deliver a specific defense-related transcriptomic response. This large-scale transcriptional reprogramming of the cell includes activation of regulatory genes in the biosynthesis of defense signaling metabolites, such as JA and SA (e.g., Glazebrook et al. 2003).

2.3 Generation of the Mobile Signals

The above paragraphs have dealt with local elicitation of resistance responses in the damaged tissue, but what is the nature of the systemically transported signal? A role in the signaling network leading to systemic IR has been unambiguously shown for only a handful of chemicals. These are hormones, such as JA and SA and their derivatives, ethylene, small RNA molecules, and VOCs.

As we have discussed elsewhere, to fully qualify as a long-distance signal, a factor must (1) induce a defensive response, (2) be produced or released at the site of attack, (3) be translocated from the attacked to the systemic tissue, and (4) accumulate in the systemic tissue before resistance expression takes place (Heil and Ton 2008). In the following section, we will discuss compounds that have received solid empirical evidence to support a function as long-distance signal.

2.3.1 Chemical Long-Distance Signals During SAR

As already mentioned (Sect. 1.2), the first convincing evidence for long-distance regulation of disease resistance in plants came from Frank Ross (1961). In subsequent decades, SAR has been studied extensively in tobacco, cucumber, bean, and Arabidopsis. An important contribution to this research was the discovery of pathogenesis-related proteins (for review, see Van Loon 1997).

It then became clear that basal resistance, PR protein expression, and SAR depend on functioning synthesis of SA and the regulatory protein NPR1 (Ryals et al. 1996; Durrant and Dong 2004). This relationship between basal resistance and SAR is also reflected by the fact that SAR is predominately effective against pathogens that are restricted by SA-dependent basal defense mechanisms (Ton et al. 2002). Hence, SAR seems to entail a systemic enhancement of SA-dependent basal resistance.

In 1990, two research groups independently reported that levels of SA increase in the vascular tissues of pathogen-infected leaves of tobacco and cucumber before the expression of SAR in the upper leaves (Malamy et al. 1990; Métraux et al. 1990), suggesting that SA acts as the long-distance signal. Labeling studies then demonstrated SA transport from pathogen-infected leaves to uninfected SAR expressing leaves of tobacco or cucumber (Shulaev et al. 1995; Mölders et al. 1996). However, these findings did not demonstrate that SAR critically depends on systemic transport of SA, and the role of SA as a systemically transported long-distance signal has been controversial for a long time.

Recently, much progress has been made in the identification of the mobile SAR signals and it has been demonstrated that they may actually differ among plant–pathogen combinations. In fact, Rasmussen et al. (1991) reported that removal of the pathogen-inoculated cucumber leaf before levels of SA in the phloem rise still conferred SAR in the upper leaves. Moreover, grafting experiments revealed that SA-deficient rootstocks expressing SA hydroxylase are capable of generating a SAR signal (Vernooij et al. 1994), suggesting that SA by itself is not the critical long-distance signal. A significant discovery was made by Shulaev et al. (1997), who found that the methylated form of SA, methyl salicylic acid (MeSA), has the ability to induce resistance to TMV in uninfected plant parts and even in neighboring tobacco plants. The importance of MeSA for SAR was further clarified by Kumar and Klessig (2003), who discovered the tobacco SA-binding protein (SABP2), which converts biologically inactive MeSA into active SA. Finally, Park et al. (2007) demonstrated, through grafting experiments with tobacco plants silenced in

expression of *SABP2* or *SA methyltransferase1* (*SAMT1*), that MeSA, and not SA, acts as the critical long-distance signal for SAR in tobacco.

Whether MeSA also functions as a critical SAR signal in other plant species remains, however, debatable. In Arabidopsis, for instance, lipid-based molecules might function as long-distance SAR regulators. First convincing evidence for involvement of lipid-derived long-distance signals came from the identification of the Arabidopsis *dir1-1* mutant (*defective in IR*: Maldonado et al. 2002). Petiole exudates from pathogen-infected *dir1* leaves failed to trigger *PR-1* gene expression, whereas exudates from infected wild-type leaves did. Because DIR1 encodes an apoplastic lipid transfer protein, it was hypothesized that DIR1 may interact with a lipid-derived molecule to mediate long-distance signaling. In addition, JA, and not SA, rapidly accumulated in petiole exudates of pathogen-infected Arabidopsis leaves, and SAR was attenuated in the JA response mutant *jin1* and the JA biosynthesis mutant *opr3* (Truman et al. 2007). However, other studies found that the JA response mutants *coi1* and *jar1* of Arabidopsis are not impaired in their SAR expression (Cui et al. 2005; Mishina and Zeier 2007). A significant break-through in the search for the long-distance SAR signal in Arabidopsis came from Chaturvedi et al. (2008), who provided compelling evidence for involvement of ω3-fatty acids. Petiole exudates from infected leaves of mutants in the biosynthesis of unsaturated galacto- and sulfoglycerolipids failed to trigger SAR in wild-type plants. Interestingly, the SAR response could be induced when the petiole exudates from these ω3-fatty acid mutants were mixed with petiole exudates from the *dir1* mutant, which alone failed to express SAR. Hence, the lipid-transfer protein DIR1 mediates long-distance transport of unsaturated galacto- and sulfoglycerolipids that activate downstream SAR signaling in systemic leaves of Arabidopsis.

2.3.2 Chemical Signals in the WR

The first and classic example of a plant WR is systemic PI synthesis in tomato (Green and Ryan1972). An 18-amino acid peptide, systemin, was identified, which is released upon feeding by chewing herbivores and is mobile within the plant (Narváez-Vásquez et al. 1995). However, grafting experiments – particularly with the systemin response tomato mutant *spr1* (Lee and Howe 2003) – have revealed that jasmonates, rather than systemin, function as long-distance signals for the systemic WR (see box 3 in Heil and Ton 2008).

What is the cumulative evidence that jasmonates are critical long-distance signals to mediate the systemic WR? First, exogenous application of JA activates all defensive mechanisms characteristic for the WR such as PIs, the release of volatiles, nicotine production, and EFN secretion (Farmer et al. 2003; Wasternack 2007; Heil 2008). Second, the rise of endogenous JA in locally damaged tissue precedes the induction of JA-inducible defenses in systemic tissues (Baldwin et al. 1997; Heil et al. 2001) and labeling studies confirmed that ^{14}C-JA and ^{11}C-methyl jasmonate are transported through the vascular system (Zhang and Baldwin 1997; Thorpe et al. 2007). Finally, grafting experiments with tomato demonstrated that biosynthesis of

JA is required at the site of damage, whereas its perception is required in the distal plant parts. For instance, wild-type scions grafted on damaged rootstocks of the *jasmonate-insensitive 1* mutant (*jai1*; impaired in JA perception but not its synthesis) were still capable of expressing PIs, while *jai1* scions grafted on a damaged wild-type rootstock failed to activate the defense response. Correspondingly, grafts with JA-biosynthetic mutants, such as the *suppressor of prosystemin 2* mutants (*spr2*) (Stratmann 2003) and the *acyl-coA oxidase1* mutant (*acx1*) (Li et al. 2005) revealed that intact JA synthesis in the rootstock, and not in the scion, is required for a functioning signal transmission (Schilmiller and Howe 2005).

Several characteristics make jasmonates optimal long-distance signals. First, JA synthesis starts with the liberation of linolenic acid from membranes followed by a multistep conversion into JA by several pre-existing enzymes (Wasternack 2007) and is, therefore, most likely controlled at the level of substrate availability (Wang et al. 1999; Laudert et al. 2000). Second, the positive correlation between damage intensity and endogenous JA concentrations (Heil et al. 2001) ensures a dose-responsive resistance induction. Third, jasmonates induce the expression of genes required for their own synthesis. Several of these biosynthetic genes are expressed in the phloem, thereby allowing for amplification of the signal during its transport (Stenzel et al. 2003, see Fig. 1). In other words, JA can amplify its own synthesis in the vascular bundles (Wasternack et al. 2006), which counteracts dilution of the signal during long-distance transport. Because labeling studies revealed that a relatively low percentage of exogenously applied jasmonates arrives at the systemic target organs (Zhang and Baldwin 1997; Thorpe et al. 2007), it is tempting to speculate that long-distance signaling by jasmonates is conceptually similar to the propagation of action potentials in neurons: A locally initiated signal triggers JA synthesis in the neighboring sector of the phloem, and the spread of this induction event functions, at least partly, as the mobile signal.

2.3.3 Electrical Signals in Herbivore Resistance

The first hints on systemic information transfer in plants were published approximately 100 years ago by Jagadis Chunder Bose and suggested electrical action potential-like signals, rather than chemical signals (Bose 1902, 1913). The concept of nervous systems in plants, however, was regarded as an inadequate anthropomorphism, a concern that significantly hindered research into a field that recently has been re-introduced as “plant neurobiology” (Brenner et al. 2006). In fact, electrical action potentials are involved in the WR of tomato. Plants dispose of two different types of electrical long-distance signals (Brenner et al. 2006): directionally propagated action potentials move trough vascular bundles along the plant axis, while slow wave potentials follow hydraulic pressure changes that move through the xylem, perhaps activating mechanosensitive ion channels in neighboring cells (Stankovic and Davies 1996). Both types of signals are elicited by mechanical or flame wounding or by direct electrical stimulation and have been shown to induce PI synthesis in tomato (Stankovic and Davies 1996). Follow-up studies

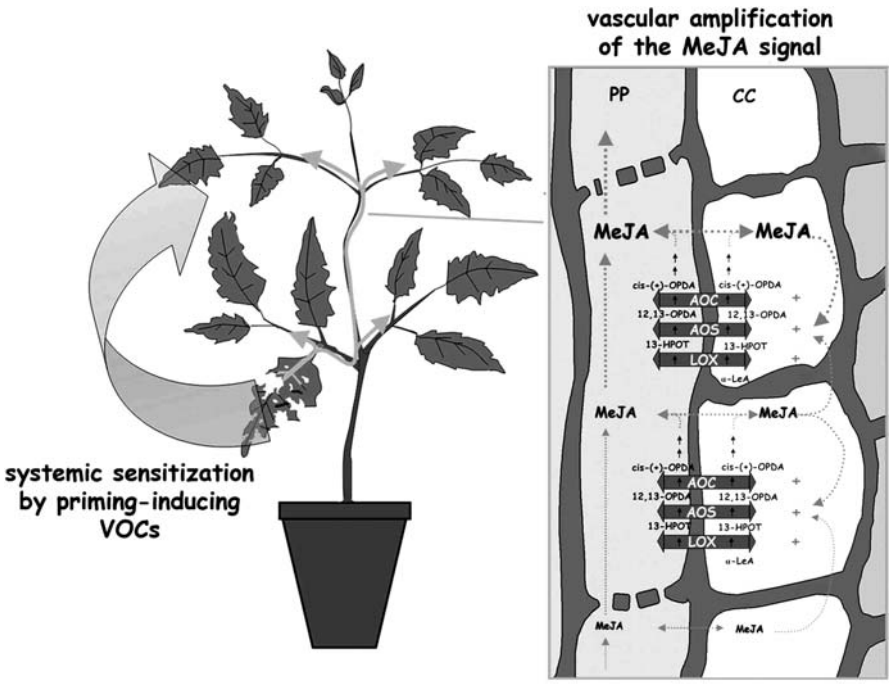

Fig. 1 Two strategies by which plants cope with dilution of the systemic wound signal. Jasmonic acid or methyljasmonate (MeJA) in phloem parenchyma (PP) cells can diffuse to the companion cells (CC), where they promote their own biosynthesis through activation of the biosynthetic genes lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cylcase (AOC). The corresponding mRNAs and/or enzymes are distributed within the PP–CC complex by transport through plasmodesmata. Secondly, locally released herbivore-induced volatiles (see also Fig. 2) can prime systemic leaves for enhanced responsiveness to the vascular MeJA signal. Both mechanisms act in synergy to mediate an optimal systemic defense response in the plant

that used direct electrical stimulation even demonstrated that action potentials alone were able to induce PI expression in a dose-responsive manner (Stankovic and Davies 1996).

2.4 Resistance Expression in the Systemic Organs

2.4.1 Signal Perception

JA, SA, their derivatives (MeJA and MeSA), lipid signals, and action potentials can move from stressed tissues throughout the plant to trigger systemic resistance responses. How are these mobile signals perceived in the systemic organs?

During SAR in tobacco, systemically transported MeSA is converted into active SA (Park et al. 2007). A critical regulator in this part of the SAR pathway is the protein NPR1 (Cao et al. 1994; Delaney et al. 1995). Mutations in *NPR1* cause insensitivity to SA, thereby blocking the induction of SA-dependent *PR* genes and SAR (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). Over-expression of NPR1 fails to activate *PR-1*, suggesting that NPR1 requires posttranslational modification to deliver the SAR signal (Cao et al. 1998). Indeed, SA-induced fluctuations in the cellular redox state promote reduction of inactive NPR1 oligomers into active monomers, which translocate to the nucleus where they stimulate *PR* gene expression (Mou et al. 2003). However, NPR1 does not directly bind to the DNA promoters of *PR* genes (Cao et al. 1997), but rather acts by recruiting and activating defense-related transcription factors (Dong 2004). In addition to regulation of *PR* genes and other defense-related transcription factor genes, NPR1 also activates genes that are involved in the protein secretory pathway, thereby ensuring proper processing and secretion of PR proteins (Wang et al. 2005).

In the context of the WR, it was recently discovered that jasmonates (most likely the JA-amino acid conjugate jasmonoyl–isoleucine) interact with the COI1 (coronatin-insensitive 1)-unit of an E3 ubiquitin ligase complex termed SCF^{COI1} (Skip/Cullin/Fbox – COI1). This event promotes binding of the COI1-unit to JAZ (jasmonate ZIM-domain) proteins, thereby targeting the JAZ proteins for ubiquitination and rapid degradation. Because JAZ proteins are repressors of JA-inducible gene expression, their degradation allows transcription factors to stimulate JA-inducible gene expression (Chini et al. 2007; Thines et al. 2007; Staswick 2008).

2.4.2 Crosstalk and Trade-offs

In nature, plants are rarely attacked by only one enemy. For that reason, plants have evolved sophisticated fine-tuning mechanisms to mount an adequate defense response against combinations of different biotic and abiotic threats. As discussed above, the plant hormones SA and JA are key players in local and systemic defense responses (Van Poecke and Dicke 2003; Durrant and Dong 2004; Heil and Ton 2008). In addition to these hormones, ethylene (Van Loon et al. 2006; von Dahl and Baldwin 2007), abscisic acid (ABA) (Mauch-Mani and Mauch 2005), brassinosteroids (Nakashita et al. 2003), and auxins (Navarro et al. 2006) may modulate plant defense responses. Genome-wide expression profiling of various *Arabidopsis*-attacker combinations revealed substantial interactions between SA- and JA-dependent defense pathways (Glazebrook et al. 2003; De Vos et al. 2005). It is thought that this pathway cross-talk provides a powerful regulatory potential that allows the plant to fine-tune its defense responses to the specific attacker encountered (Korneef and Pieterse 2008).

A well-studied example of signaling cross-talk is the antagonistic interaction between SA and JA (Spoel et al. 2003; Bostock 2005). Although synergistic interactions have been described (Van Wees et al. 2000; Mur et al. 2006), most reports indicate “trade-offs” causing a suppression of JA-dependent defense traits

in plants expressing SA-dependent resistance or being treated with exogenous SA (Bostock 2005; Korneef and Pieterse 2008). One may wonder why this is the dominant direction of the cross-talk, as herbivory generally poses a much more immediate threat than slowly colonizing pathogens: an observation that might lead to the ad hoc expectation that defense against herbivores should dominate over pathogen resistance. Among other possible explanations, plants generally recover faster from herbivory than from infection by biotrophic pathogens. In addition, many herbivores are vectoring pathogens to the plants: herbivore feeding has, thus, a high predictive value for future pathogen infection (Heil and Bostock 2002).

Interestingly, Spoel et al. (2007) recently discovered that the negative cross-talk between *Pseudomonas syringae*-induced SA and JA-dependent resistance against the necrotrophic fungus *Alternaria brassicicola* only occurred when both pathogens were attacking the same leaf. *P. syringae* failed to suppress resistance against the necrotrophic fungus in the systemic leaves. Hence, SA-inducible suppression of JA-dependent resistance is not regulated by SAR-related long-distance signals.

Trade-offs also exist between plant defense and primary metabolism. Defense traits are costly and plants can invest their limited resources into either growth or defense, but not both. In fact, inducible defense traits are, at least partially, regarded as a cost-saving strategy (Heil and Baldwin 2002). Furthermore, plants can potentially increase their fitness by reducing defensive investments under conditions when future competition with other plants is likely (Cipollini 2004). This future competition can be sensed by the quality of light reaching the plant's shoot, which is particularly exposed to lateral light. Light passing through a canopy contains relatively little red and blue light, but is enriched in far-red light. Plants have specific phytochrome receptors for these wavelengths and perceive the quality and quantity of light to avoid being shaded by neighbors (Ballaré and Scopel 1997; Ballaré 1999). Recently, a trade-off between far-red sensing and defense induction was reported in the South American native tobacco species, *Nicotiana longiflora*: when far-red light signaled the presence of putative competitors, the plants suppressed defense expression even when being under attack (Izaguirre et al. 2006).

3 Airborne Signaling

PI expression in tomato has been observed in response to both JA (Sect. 2.3.2) and electrical signaling (Sect. 2.3.3), illustrating that a single plant can use more than one type of signal to regulate systemic defense. In fact, the spectrum of systemic defense signals has recently been widened with VOCs. These airborne compounds represent a novel class of signals that can regulate systemic plant responses in response to local enemy attack (Fig. 2).

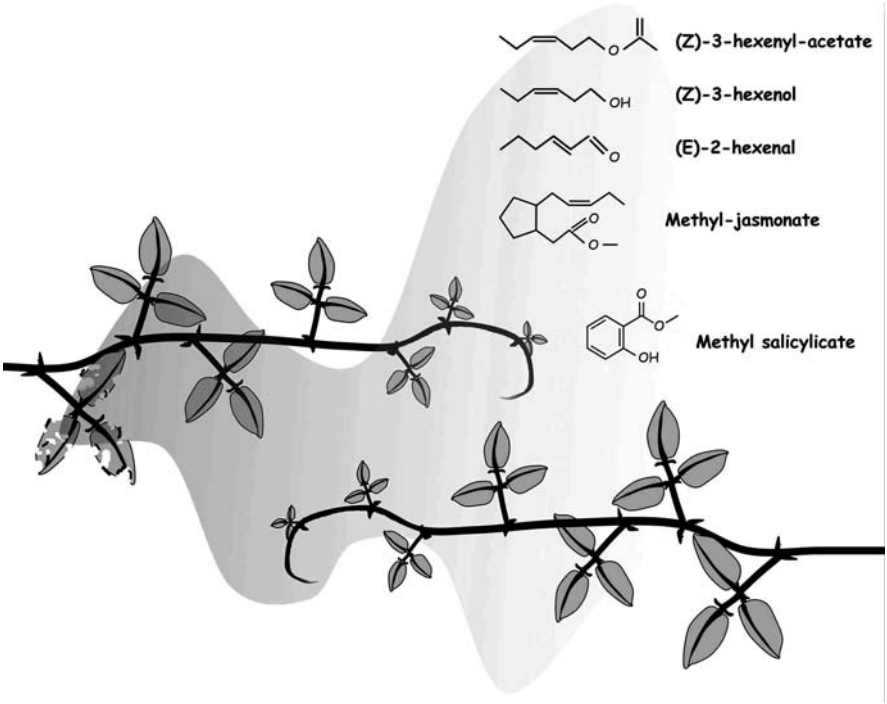

Fig. 2 Between- and within-plant signaling by airborne signals. Localized herbivore attack triggers a complex blend of volatile organic compounds (VOCs), which can induce resistance in systemic tissues and nearby plants. Oxylipins, such as methyljasmonate, (Z)-3-hexenylacetate, (Z)-3-hexenol, and (E)-2-hexenal, as well as aromatic volatiles, such as methylsalicylate, can trigger defensive reactions at relatively high concentrations. At lower concentrations, these VOCs can prime the plant tissues for an augmented defense reaction upon subsequent herbivore attack

3.1 A Short Historical Overview

Airborne signaling has mostly been described in a context of signaling between plants. David Rhoades (1983) was the first to report that sitka willow (*Salix sitchensis*) trees in close vicinity to caterpillar-infested individuals expressed enhanced resistance to tent caterpillars (*Malacocoma californicum*). Since then, the phenomenon has been described for various plant species, such as *Arabidopsis thaliana*, black alder (*Alnus glutinosa*), corn (*Zea mays*), lima bean (*P. lunatus*), poplar (*Populus euroamericana*), sagebrush (*Artemisia tridentata*), and wild and cultivated tobacco (*N. attenuata* and *N. tabacum*) (Baldwin and Schultz 1983; Shulaev et al. 1997; Karban et al. 2000; Engelberth et al. 2004; Kost and Heil 2006; Ton et al. 2007; Godard et al. 2008). Most of these cases reported signaling between conspecific plants. However, clipped sagebrush has been shown to induce resistance in neighboring wild tobacco (Karban et al. 2000).

While the first reports about plant–plant communication were criticized for their lack of ecological realism (Baldwin and Schultz 1983) or their lack of true controls (Rhoades 1983), later experiments confirmed that black alder trees growing downwind of clipped individuals developed resistance against herbivores (Tscharntke et al. 2001). Similarly, field studies with lima bean (*Phaseolus lunatus*) demonstrated that otherwise untreated receivers suffer less from herbivory when exposed to VOCs from beetle-damaged emitters (Heil and Silva Bueno 2007) and that they develop resistance to *P. syringae* when exposed to the air released from SAR expressing neighbors (Yi et al., unpublished data). Hence, plant–plant communication is a phenomenon that provides genuine protection under ecologically realistic conditions!

3.2 Within-Plant Signaling by VOCs

3.2.1 Airborne Within-Plant Signaling: The Phenomenon

Airborne plant–plant communication mostly benefits the receiving plant, which uses these signals to adapt its defensive capacity according to the upcoming threat. Since plants compete for light, space, water, and nutrients, this communication may have detrimental effects for the emitting plant, which is already damaged. Why would plants warn their neighbors? In an attempt to answer this question, Edward E. Farmer and Colin Orians (Farmer 2001; Orians 2005) speculated that VOCs might also serve as within-plant signals. This hypothesis has been supported by experiments with mechanically damaged sagebrush (*A. tridentata*), which revealed that airflow within the plant is essential to obtain systemic IR against herbivores (Karban et al. 2006). Similarly, systemic induction of EFN secretion only occurred in wild lima bean if air could move freely from attacked to undamaged leaves (Heil and Silva Bueno 2007), and volatiles from herbivore-damaged poplar leaves (*Populus deltoides x nigra*) augmented defense reactions in adjacent leaves within the same individual (Frost et al. 2007).

In the classical case of chemical or electrical signaling via the vascular system, damage of a single leaf elicits the strongest systemic responses in orthostichous leaves (Orians 2005). Resistance to herbivores in poplar (Davis et al. 1991), tomato (Orians et al. 2000), tobacco (Schittko and Baldwin 2003), and *Trifolium repens* (Gómez and Stuefer 2006) followed the spatial pattern of assimilate transport and seems, therefore, confined to the phloem. Likewise, girdling of the petioles of pathogen-infected cucumber leaves blocked SAR development, indicating signaling through the phloem (Guedes et al. 1980). However, systemic resistance can also be activated in distal leaves that lack a direct vascular connection to the attacked leaf (Mutikainen et al. 1996; Kiefer and Slusarenko 2003). In these cases, airborne signaling by VOCs seems the most likely explanation for the systemic resistance.

3.2.2 Airborne Within-Plant Signaling: The Mechanism?

Bio-active VOCs triggering defense in plants are still being discovered, but most reports on airborne signals that lead to signaling between plants and that likely also cause within-plant signaling via the plant's headspace concern gaseous derivatives of JA and SA (MeJA and MeSA) and green-leaf volatiles (GLVs) (Arimura et al. 2000; Engelberth et al. 2004; Ruther and Fürstenau 2005; Ruther and Kleier 2005; Kost and Heil 2006). Because GLVs are synthesized by pre-existing enzymes from precursors that are already present in undamaged cells, they are rapidly released upon tissue damage (Turlings and Wäckers 2004). Typical GLVs that prime or induce herbivore resistance are *cis*-3-hexenyl acetate (corn and lima bean: see Engelberth et al. 2004; Kost and Heil 2006; Heil et al. 2008) and *cis*-3-hexen-1-ol, *trans*-2-hexenal, *cis*-3-hexenal, *trans*-2-pentenal, and *trans*-2-heptenal (corn: see Engelberth et al. 2004; Ruther and Fürstenau 2005).

In addition to jasmonates, MeSA and GLVs, the gaseous hormone, ethylene, can act as an airborne signal. Ethylene plays a modulating role in plant defensive reactions to pathogens (Van Loon et al. 2006) and herbivores (Xu et al. 1994; von Dahl and Baldwin 2007). For instance, ethylene boosts *cis*-3-hexenol-induced emission of sesquiterpenes in maize, even though ethylene alone had no effect on the release of these volatiles (Ruther and Kleier 2005). Hence, ethylene potentiates the plant's response to GLVs, but does not necessarily serve as a primary signal itself.

How do airborne signals trigger systemic IR? Both MeSA and MeJA can be converted into SA and JA, respectively, which provides an obvious physiological explanation for plant responses to these two volatiles. In contrast, little is known about the physiological impacts of GLVs. Although it has been suggested that GLVs with an α, β-unsaturated carbonyl group can trigger defense through their activity as reactive electrophile species (Almeras et al. 2003), there are examples of biologically active GLVs that lack this motif (Ruther and Kleier 2005; Kost and Heil 2006; Heil et al. 2008). Changes in transmembrane potentials are involved in early signaling events in the cellular response to stress (Maffei et al. 2007a). Furthermore, exposure to GLVs, such as *cis*-3-hexenyl acetate, can change membrane potentials in intact lima bean leaves (Massimo Maffei, personal communication). Considering the role of action potentials in the induction of herbivore resistance, integration of small lipophilic compounds into the plasmembrane might lead to changes in transmembrane potential, or somehow affect membrane integrity. This could result in a direct elicitation of cellular defense pathways or mediate an enhanced responsiveness of the cell to PAMPs, MAMPs, or HAMPs. Above all, much more research is required before we understand the mechanisms by which plants perceive GLVs or other resistance-inducing plant odors.

3.2.3 Priming by Airborne Signals

Priming by volatiles is emerging as a major topic in plant–herbivore and plant–plant interactions (Baldwin et al. 2006; Turlings and Ton 2006). Analogous to

chemicals, such as isonicotinic acid (INA) and benzothiadiazole (BTH), herbivore-induced volatiles at lower concentrations prime plant tissues, rather than evoking a direct defense response (Engelberth et al. 2004; Baldwin et al. 2006; Turlings and Ton 2006). Laboratory studies with maize revealed that herbivore-induced volatiles can prime neighboring plants for enhanced expression of direct and indirect defenses, resulting in reduced performance of Egyptian cotton leafworm (*Spodoptera littoralis*) caterpillars and improved attractiveness to parasitoid *Cotesia marginiventris* wasps (Ton et al. 2007). Importantly, herbivory-induced volatiles have also been demonstrated to prime nearby plants under natural conditions (Kessler et al. 2006; Heil and Silva Bueno 2007). Volatile-induced priming seems a common defense strategy in plants as it functions as a cost-efficient resistance mechanism to prepare the systemic tissues for upcoming attack.

3.2.4 Molecular and Physiological Mechanisms of Priming

Priming is not always easy to study in a molecular context, since most priming phenomenona are not clearly characterized by molecular markers. Much recent progress in the molecular understanding of priming comes from the response of *A. thaliana* to priming chemicals, such as BTH and beta-aminobutyric acid (BABA) (Conrath et al. 2006). It is thought that the primed defense state is based on an enhanced expression of signaling proteins that remain inactive until the plant is exposed to stress. Upon perception of a second, stress-derived, signal, the augmented pool of signaling proteins would allow for a faster and stronger defense reaction in the plant.

Recent evidence supports this hypothesis. For instance, increased expression of the inactive form of MAPK3 has been reported in response to BTH, but the kinase required a second pathogen-derived signal to become activated (Beckers and Conrath 2007). Furthermore, Van der Ent (2008) reported that priming by non-pathogenic rhizobacteria or BABA leads to enhanced expression of defense-related transcription factors without major induction of defense-related genes. Hence, priming indeed is associated with an increase in the pool of defense-related signaling proteins. It seems, however, likely that additional regulatory mechanisms are involved. Recently, Bruce et al. (2007) proposed involvement of epigenetically related regulatory mechanisms in priming. In support of this, prelimimary results indicate that chemically induced priming in Arabidopsis promotes the formation of open chromatin structures around the promoter region of the defense-related marker gene *PR-1* (J. Ton, unpublished results). Apart from such chromatin remodeling effects, it is not unthinkable that priming is accompanied with changes in DNA methylation. Such epigenetically related modifications would allow for a facilitated access of transcription factor proteins to *cis*-acting regulatory motifs of defense-related genes. Moreover, epigenetic stress-imprints could account for the long-term effects of priming. Understanding how far these mechanisms influence the kinetics of defense activation upon pathogen/insect attack remains a major challenge for the future.

3.2.5 Benefits, Shortcomings and Synergy Between Vascular and Airborne Signaling

Plants possess a well-developed vascular system, through which both chemical and electrical signals can be transported. Why would plants, then, use volatiles for long-distance defense signaling? The most apparent shortcoming of airborne signaling is the possibility of eavesdropping: competing neighbors can make use of the information that a plant is infested and prepare for this threat before they are attacked themselves. Secondly, wind affects the direction, concentration and velocity at which the signals are distributed: airborne signaling certainly comes with the price of limited controllability by the plant. Why has airborne signaling evolved at all? We propose that systemic signaling by VOCs provides plants with several important benefits.

(1) First, internally transported signals are restricted to the vascular architecture of the plant. Phloem-mobile signals are predominantly bound to source-sink relations, whereas xylem-mobile signals are restricted by the direction of the upwards transpiration flow (Davis et al. 1991; Orians et al. 2000; Orians 2005; Gómez and Stuefer 2006). Even in cases where the distributional patterns of resistance expression can only be explained by bidirectional transport (Schittko and Baldwin 2003; Thorpe et al. 2007), the IR could only be elicited in orthostichous leaves. Therefore, airborne transportation complements the restriction of vascular signaling: VOCs can serve as a cue to trigger defense responses in those plant parts where resistance is mostly required: in the spatially (yet not necessarily anatomically) neighboring parts.

(2) In the second place, airborne transportation of signals is considerably faster than vascular transport. Long-distance transport of chemical signals through vascular tissues can last hours before reaching the systemic tissues, whereas diffusion by airborne signals occurs in seconds. Since timing is crucial for the effectiveness of induced defense, airborne defense signals may in some interactions be far more important than vascular signals.

(3) Thirdly, airborne and vascular defense signals can act in synergy. Since self-priming by herbivore-induced volatiles has been described in the context of airborne within-plant signaling (Frost et al. 2007; Heil and Silva Bueno 2007), we propose a two-step regulatory system, in which airborne signals prime distal tissues to respond more efficiently to vascular signals or direct attack (Heil and Ton 2008). According to this model, the systemic tissues would only respond with a full defense response when the airborne priming signal is confirmed by the vascular signal (Fig. 1). This allows for additional fine-tuning of the systemic defense response, in which the airborne signal tailors the plant's systemic defense response to the amount of local damage.

(4) Finally, dosage-dependent effects should strongly reduce the putative ecological cost of external signaling. Volatile-mediated signaling works most efficiently over relatively short distances, and the probability that the leaf nearest to an attacked one belongs to the same plant is high. As a result, the chance that

eavesdropping by competing plants becomes a quantitatively relevant problem remains relatively low (Heil and Ton 2008).

3.2.6 How Far Do External or Internal Signals Travel?

As discussed above, internally transported chemical signals move slowly and are dependent on the vascular architecture of the plant. Especially in larger plants, such as trees and shrubs, dilution to inactive doses can pose a realistic limitation to the effectiveness of internal signaling. Despite these limitations, however, internal signals have been shown to travel over relatively long distances (Van Bel and Gaupels 2004). There are two likely explanations for this long-ranging effectiveness. As mentioned above for jasmonates (Sect. 2.3.2), long-distance plant signals can induce the expression of genes that promote their own synthesis. For instance, primary pathogen infection of a single cucumber leaf triggered transient increases in activity of the SA biosynthesis enzyme PAL in stems and petioles that preceded a detectable increase in SA (Smith-Becker et al. 1998), suggesting that SA or MeSA is synthesized de novo in stems and petioles during the process of long-distance transport, thereby counteracting dilution of the signal. Secondly, signal dilution can be prevented by volatile-mediated priming of the systemic tissues to achieve an enhanced responsiveness to the internal signal (Heil and Ton 2008). Therefore, we propose that the effectiveness of long-distance internal plant signals depends on (1) de novo signal biosynthesis in the vascular tissues and (2) self-priming of systemic tissues by early acting airborne signals (Fig. 1).

Distance is also a crucial parameter in plant–plant communication, and positive reactions are restricted to the close vicinity to the emitter. Heil and Silva Bueno (2007) had intertwined senders and receivers to mimic the natural growth of lima bean, a liana. Resistance induction was also found in wild tobacco plants growing 15 cm downwind from clipped sagebrushes (Karban et al. 2000) and in black alders growing at a distance of 1 m from clipped trees (Tscharntke et al. 2001). The distance over which airborne cues can affect other plants depends strongly on wind speed, air humidity, and temperature, but in general it appears to be very short.

4 Below and Aboveground Interactions

4.1 Herbivore Feeding on Shoots and Roots

Plants are important mediators in the communication between above and belowground ecosystems and can, as such, influence entire food webs. Consequently, dynamic interactions between below and aboveground plant defenses can have far-reaching impacts at different trophic levels. Several reviews have been published on below–aboveground interactions (Blossey and Hunt-Joshi 2003; Wardle et al. 2004; Bezemer and Van Dam 2005). Nevertheless, the ecological function of these

interactions remains unclear. Are below–aboveground interactions of plant defenses beneficial to the plant or to the attacker? Or are there no clear-cut benefits? Apart from these ecological questions, the physiological basis of below and aboveground defense interactions is poorly understood (Erb et al. 2008). Preliminary results point to a possible role for ABA as a long-distance signal behind below and aboveground defense interactions in maize: Damage by the root herbivore *Diabrotica virgifera* significantly boosted ABA accumulation and ABA-inducible gene expression in the leaves, which coincided with IR to aboveground pathogens and herbivores (Erb et al. 2009). It is tempting to speculate that these ABA effects are due to host manipulation by the root herbivore to promote transport of photo-assimilates to the roots. For instance, a decreased water potential in the roots would not only boost ABA transport from the roots to the shoot, but it would also stimulate sugar transport to the roots along the osmotic pressure-gradient that drives phloem transport.

4.2 Signaling Between Plants and Beneficial Microorganisms

Apart from pathogens, there are microorganisms that live inside plant tissues without harming them. These fungal and bacterial microorganisms are called endophytes. Because many endophytes are soilborne, they mostly colonize plant roots. Specialist examples of endophytes are mycorrhizal fungi and root-nodulating bacteria. Additionally, there are many endophytic fungi and bacteria with broader host ranges, of which some are known to exert plant-beneficial effects, such as growth promotion and induced systemic resistance (ISR) against biotic and abiotic stress. Although beneficial microbes are specialized to colonize plants, they never reach population sizes that are damaging the host plant. In combination with the fact that rhizosphere-colonizing and endophytic microbes possess MAMPs, it is likely that the host plant expresses mechanisms to constrain growth of the plant-beneficial microbes, thereby preventing escalation into a hostile relationship.

The majority of generalist rhizosphere-colonizing and endophytic microorganisms require oxygen (O_2) for their primary metabolism. Because O_2 levels in and around roots can drop below ambient (Geigenberger 2003; Van Dongen et al. 2008), root colonization by aerobic microbes is limited by hypoxic conditions. Like all aerobic organisms, rhizosphere-colonizing and endophytic microbes respond to hypoxia by activating fermentation pathways. By degrading hexoses to ethanol and/or lactic acid, NADH is recycled into NAD+, which can then be reused in the glycolysis. Because ethanol and lactic acid are potentially toxic, many bacteria have acquired the ability to switch to the 2,3-butanediol fermentation pathway in response to declining O_2 and pH. Interestingly, the final product of this fermentation pathway is the volatile compound 2,3-butanediol, which can induce growth promotion and disease resistance in Arabidopsis (Ryu et al. 2003, 2004). As such, 2,3-butanediol can be regarded as a signaling compound in the communication between plants and plant-beneficial microbes.

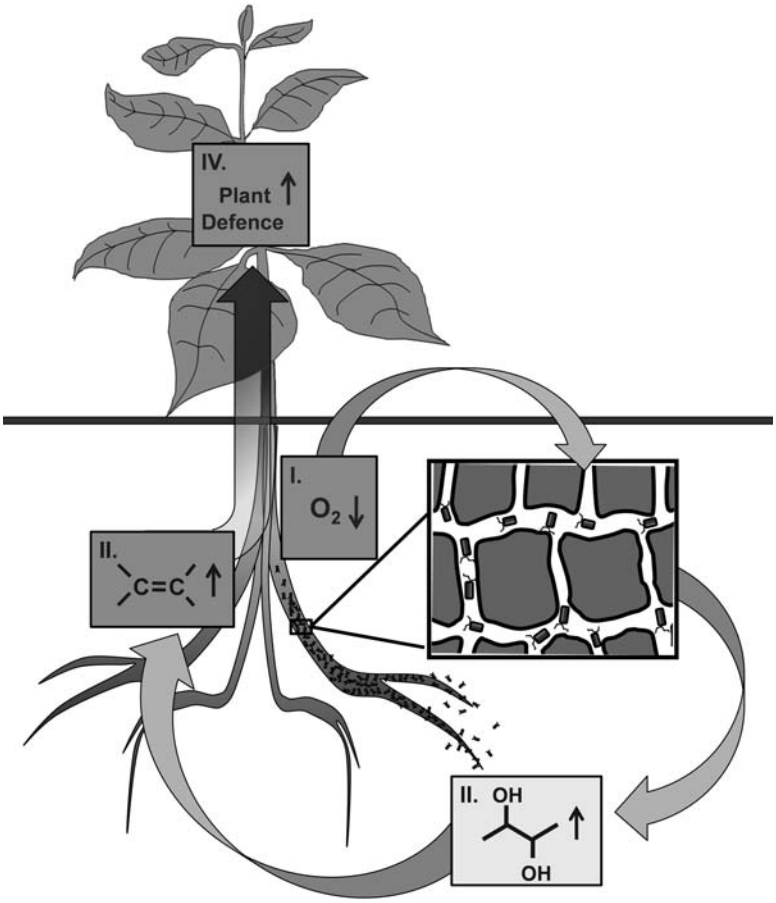

Fig. 3 Proposed signaling interplay between plants and rhizosphere-colonizing and/or endophytic microbes. The plant consumes O_2 by respiration, leading to hypoxic conditions in and around nonphotosynthetic tissues, such as roots (stage I). Microbes can adapt to these conditions by activating the 2,3-butanediol fermentation pathway (stage II). In response to 2,3-butanediol, the host plant activates ethylene-dependent processes (stage III). Besides changes in plant growth and metabolism, ethylene also activates an enhanced defensive state in the colonized tissues to control the population of plant-beneficial microbes (stage IV). Hence, self-imposed hypoxia enables plants to localize and constraint rhizosphere-colonizing and/or endophytic microbes

Apart from the low O_2 availability in plants, endophytic bacteria are restricted by a defensive system that is controlled by ethylene (Iniguez et al. 2005). Interestingly, this gaseous hormone has also been implicated in rhizobacteria-mediated ISR and 2,3-butanediol-IR in Arabidopsis (Pieterse et al. 1998; Ryu et al. 2004). Hence, ethylene not only controls microbe-IR, it also adjusts the plant's metabolism to the hypoxia that triggers 2,3-butanediol production in the microbes. Ethylene signaling in the host and 2,3-butanediol production by the microbe may well function

as important signaling components to keep the plant–microbe interaction mutualistic (Fig. 3).

5 Conclusions and Outlook

Much has been learned since the earliest discoveries of systemic resistance responses to local virus infection (Ross 1961) or herbivore attack (Green and Ryan 1972). Major signaling cascades have been detected that underlie the local perception of pathogens and herbivores. In addition, hundreds of genes have been identified that mark or regulate local and systemic defense responses. This knowledge has greatly facilitated the search for critical long-distance signals in systemic resistance responses. Since the earliest discoveries of "talking trees" (Baldwin and Schultz 1983; Rhoades 1983), it also became clear that systemic signaling does not stop at the plant surface, but that it can even affect neighboring plants.

Despite these exciting developments, there are still many questions that need to be answered before understanding the ecology and evolution of IR, and before IR can be exploited as a reliable, environmentally friendly strategy for crop protection. In both contexts, many more studies will need to quantify costs and benefits of IR traits under various ecological conditions and using plant species with different life histories. The focus of major research efforts on very few selected model species has resulted in partially contradictory results, particularly with respect to (1) whether and to which degree IR leads to fitness benefits and (2) the mobile signals that mediate systemically IR to herbivores, nectrotrophic pathogens, and biotrophic pathogens. A considerable part of these apparent contradictions might, however, just be a consequence of plants employing different strategies under different environmental conditions and of different plant species using different signals to mount phenotypically similar resistance responses.

Intense, direct activation of defenses can be costly, which makes plant defense activators unpopular for crop protection. Priming of defense, however, confers plant protection without direct induction of costly defense mechanisms, and has been shown to provide benefits to plants under conditions of relatively high disease pressure. If priming is to find a place in practical crop protection, it will be necessary to increase our understanding of the molecular, physiological and ecological aspects of the phenomenon.

Much more has to be done to disentangle the complex interplay between abiotic factors, such as light and soil nutrients, and biotic factors, such as pathogens, herbivores, interplant competition, and plant-beneficial microorganisms. All these factors have to be taken into account when exploring the broader context of IR on plant health, growth, and yield.

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Roots: The Acquisition of Water and Nutrients from the Heterogeneous Soil Environment

Angela Hodge

Contents

Abstract The distribution of water and nutrient resources in soil is heterogeneous. Plant roots have to cope with, and exploit, this heterogeneity in resource supply to acquire these two key resources. Here, the responses by roots including rooting depth, root demography, morphological and physiological plasticity to heterogeneous supplies of water and nutrients will be reviewed and their ecological consequences discussed. How roots themselves influence water distribution through the process of hydraulic redistribution and the ecological consequences of this process, including the impact on nutrient availability, will also be examined.

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1 Introduction

The main functions of plant root systems are anchorage and the acquisition, and transport, of water and nutrients. In larger plant species, specialised supporting structures may be required to anchor the plant, whereas in smaller species, this is often achieved as a consequence of normal root growth and function including nutrient foraging and transport. Roots need to forage for resources because in all soils the distribution of water and nutrients are heterogeneous at scales relevant to plant roots (Grose et al. 1996; Jackson and Caldwell 1993a). Roots must, therefore, be able to locate and exploit these resource rich zones or "patches" in order to survive. This is aided by the modular structure of roots where the number of modules is not fixed, thus allowing great flexibility (or plasticity) and the ability to respond to the prevailing environmental conditions. There has been much recent interest in the response of roots to both water and nutrient heterogeneity to assess whether these responses can be exploited in agriculture including through crop breeding of desired traits (Morison et al. 2008; Neumann 2008; de Dorlodot et al. 2007), which in turn may lead to more sustainable agricultural practises through a reduction of external inputs of water and fertiliser applications. However, if this is to be achieved, the ecological context in which the root response is evoked also needs to be understood. Thus, in this chapter, the current knowledge on how root systems respond to both water and nutrient availability will be examined with emphasis on the ecological importance of these responses. In addition, how roots themselves influence water distribution through the process of hydraulic redistribution and the ecological consequences of this process will also be discussed.

2 Roots Response to Water Availability

2.1 Rooting Depth in Water Limited Environments

The lowest water potential at which plants can access water from the soil is at their permanent wilting point. Water is available to the plant from their permanent wilting point to field capacity (the maximum water that a soil holds in its pores through surface tension after excess water has drained away). For most plants, water is available at soil matric potentials greater (i.e. less negative) than −1.5 MPa (the wilting point of many mesophytic plants; Bengough et al. 2006). However, some plants (e.g. xerophytes and halophytes) have much lower (i.e. more negative) permanent wilting points allowing water uptake at much lower soil matric potentials although water extraction becomes more difficult, as it is confined to only very fine pores and hydraulic resistance is high (Fitter and Hay 2002). Moreover, the availability of water will vary both vertically and horizontally within the soil profile. Thus, the ability to develop deep, more extensive, root systems under water stressed conditions may be advantageous (Huck et al. 1983; Price et al.

1997; Songsri et al. 2008), although, often this may be accompanied by depressed shoot growth (Huck et al. 1983). Poot and Lambers (2008) recently demonstrated that two rare *Hakea* species found in *shallow* soils in low water environments rapidly produced deep roots, which enabled water to be located in cracks in the underlying bedrock before the onset of drought and enabled these rare species to persist in these particular environments. However, both rare species had poor competitive ability when transplanted into deep soils, presumably due to the cost of deep root investment.

It may, therefore, be expected that maximum rooting depth would increase in low water environments; however, Schenk and Jackson (2002a) found in such environments *absolute* rooting depth tended to increase with mean annual precipitation (MAP) for all plant growth forms, except trees and shrubs. Thus, root systems tended to be shallower and wider under dry hot climates. As Schenk and Jackson (2002a) highlighted, this finding is seemingly counter to the "often-held assumption" that rooting depth *increases* in *drier* areas, but as these authors stress the distinction between *absolute* and *relative* rooting depths must be made clear. For a given canopy size, herbaceous plants, in fact, have greater maximum rooting depths in drier environments, even though as the canopy size increases so usually does absolute rooting depth (Schenk and Jackson 2002a). Furthermore, the *proportion* of the total root system biomass does tend to be deeper in drier environments (Schenk and Jackson 2002b) with "deep" roots (considered to be if 5% or more of all roots in a profile are located below 2 m depth) least likely to occur in arctic, boreal or cool-temperate regions and in per-humid climates, such as equatorial rain forests (Schenk and Jackson 2005). However, the distribution of water is complex and more shallow rooted species may also benefit from growing near any dimorphic rooted species, where via the process of "hydraulic redistribution" (*sensu* Burgess et al. 1998; see Sect. 4) water may be brought from wetter layers (in this case at greater depth), and released into the upper soil layers (Richards and Caldwell 1987; Caldwell and Richards 1989). Hydraulic redistribution generally occurs at night, although under some conditions, it can also occur during the day, depending on the plant species present (Yoder and Nowak 1999). Thus, plant species that have roots at depth may also benefit other members of the plant community in drier environments through the process of hydraulic redistribution, although the actual redistribution of this water is also patchy.

In addition, having a shallow yet, extensive, root system may allow exploitation of light rainfall events when they do occur. These light rain events fail to infiltrate the soil to any great depth in arid regions, thus are best exploited by having shallow roots at, or near the surface (Hunt et al. 1987). Some plant species, such as *Agave deserti* may also induce formation of ephemeral "rain" roots specifically to take advantage of such rainfall events then shed these roots during drought (Hunt et al. 1987; Nobel et al. 1990). Rainfall events may also result in transient increases in nutrient availability, particularly nitrate (Cui and Caldwell 1997) and in some cases phosphate (Campo et al. 1998), although not always (Cui and Caldwell 1997). The availability of these nutrients will depend on several factors including the dryness of the soil, the timing of the rainfall in the season and the size of the rainfall event

(Campo et al. 1998; Maestre and Reynolds 2007; James and Richards 2006; Loik 2007). Plant species also differ in their ability to acquire nutrients from such rain events (BassiriRad et al. 1999; Gebauer and Ehleringer 2000; Gebauer et al. 2002). Moreover, nitrogen (N) and water uptake may not occur simultaneously suggesting different parts of the root system are responsible for acquisition of these two key resources (Gebauer and Ehleringer 2000). Lamb et al. (2007) also recently concluded the mechanisms for competition for water and N likely differ.

2.2 Root Responses to Water Availability

When water availability is reduced, contradictory effects on root demography have been observed with both decreased (van der Weele et al. 2000; Comas et al. 2005) and increased (Reid and Renquist 1997; Jupp and Newman 1987) root production reported. While some of the differences are inevitably due to differences among plant species or the severity of water stress experienced (see van der Weele et al. 2000), the manner in which the soil dries may also be important. For example, Dubrovsky et al. (1998) found under rapid drying conditions roots of the desert succulent *Opuntia ficus-indical* showed increased mortality and decreased growth, while under more gradual drying conditions, root mortality was lower, but the length of the elongation zone and meristematic cell number decreased. While in another desert succulent (*A. deserti*), only lateral roots near the soil surface showed high mortality while new main roots were initiated (North and Nobel 1998). Thus, the part of the root system followed may also influence the results obtained. Mortality rates of fine roots following soil drying were also found to be greater in adult compared to seedling citrus (*Citrus volkameriana*) plants (Espeleta and Eissenstat 1998), thus, developmental stage may also impact upon the root demographic response observed. Mainiero and Kazda (2006) found *Fagus sylvatica* (European beech) did not alter fine root turnover in response to soil drying. However, some plants do adjust their demography in response to water availability (Green et al. 2005; Santantonio and Hermann 1985; Pregitzer et al. 1993; Peek et al. 2005) and even *Juniperus osteosperma*, a species with a relatively long root medium lifespan (ca. 1 year), demonstrated sufficient root turnover to change its overall root distribution in the soil profile to track the availability of water during the growing season (Peek et al. 2006).

Rewetting events have been found to increase root production in some studies (Lauenroth et al. 1987; North and Nobel 1998; Huang and Nobel 1993), while an increase in root death has been observed in others (Reid and Renquist 1997; Jupp and Newman 1987). Both these factors affect the root population present at a given time. New roots tend to have much higher absorption rates than older roots and their production may be essential to enable continued response to enhanced water availability (Lauenroth et al. 1987; Nobel et al. 1990). However, there is also increasing evidence, at least from some species, that older roots may play a

much larger role in water uptake than previously believed (MacFall et al. 1991; D'Hertefeldt and Jónsdóttir 1999; North and Baker 2007).

Roots typically have reduced hydraulic conductivities (i.e. reduced permeability to water) under drought conditions (Shone and Flood 1980; Huang and Nobel 1993; North and Nobel 1998; Dubrovsky et al. 1998; but see also North and Baker 2007). Reduced hydraulic conductivity helps to decrease the loss of water from the roots to the soil, which may occur if the water potential of the soil declines below that in the root (Neumann 2008). There is evidence that hydraulic conductivity can be recovered following rewetting, but the extent of the recovery depends on the root type and region of the root (North and Nobel 1991; Martre et al. 2001). The length of the drought period will also influence the extent to which root hydraulic conductivity may be restored (North and Nobel 1998). Variation in hydraulic conductance among roots is due to a number of factors including anatomical variation in root diameter, xylem structure (e.g. diameter of vessels and connectivity) and current functional state (portion of active vessels), extent of submerisation and connectivity to soil moisture (see Jackson et al. 2000; Nobel and Cui 1992; North and Nobel 1994; North and Baker 2007). The presence of aquaporins is also an important cause of variation in root hydraulic conductance (McElrone et al. 2007; North et al. 2004; North and Nobel 2000). Aquaporins are water-conducting membrane protein channels, which play a key role in recovery from drought (Javot and Maurel 2002; Martre et al. 2001; Kaldenhoff et al. 2008). Arbuscular mycorrhizal (AM) symbiosis can also influence root hydraulic properties through affecting the aquaporins in the root (Aroca et al. 2007), which may partially explain the increased drought resistance observed in mycorrhizal plants.

Root diameters often decrease under reduced water potentials (Lauenroth et al. 1987; Sharp et al. 1988; West et al. 2004; Cortina et al. 2008) due to a change towards a greater length of thinner roots, i.e. a tendency towards increased specific root length (SRL) (Sharp et al. 1988; Cortina et al. 2008). Finer roots also tend to have higher absorptive capacity (Eissenstat et al. 2000). However, the ability to penetrate soils is likely related to root diameter (Materechera et al. 1992; Clark et al. 2003). Thus, if the soil also becomes harder due to the complex interactions among soil strength, bulk density, and reduced water and oxygen availability, then it may also become more difficult for the smaller diameter roots to grow through the soil. Moreover, any radical contraction of the root system may lead to a break in the physical contact between the root and the soil substrate. This, in turn, will have an adverse impact on water movement to the root as root–soil contact is essential to aid water movement under reduced resistance (Coutts 1982).

3 Root Responses to Heterogeneous Water Supply

The distribution of water in soil is heterogeneous and can vary both horizontally and vertically at the centimetre scale within the soil profile (Dekker et al. 2001; Ritsema and Dekker 1994). Such scales are relevant to plant roots although the

presence of roots and their distribution also, in turn, impact upon the spatial heterogeneity of water in the soil profile (Grose et al. 1996; Wan et al. 1995). Although roots have long been suspected of showing a hydrotropic (i.e. growth towards water) response, due to the problems in distinguishing between hydrotropism and gravitropism, it was very difficult to demonstrate hydrotropism experimentally. Recently, however, Kobayashi et al. (2007) have identified a novel gene, *miz1,* in *Arabidopsis* that appears important for hydrotropism. Moreover, mutants of this gene showed normal gravitropism, which suggests the hydrotropism and gravitropism pathways are independent of each other, although the root cap is the detection centre for both. However, the ecological significance of root hydrotropism is still far from certain (Cole and Mahall 2006) and much more realistic experimental conditions are required before any such role can be determined.

It is established that plant roots are able to respond to, and exploit, zones or patches of water that they encounter in soil (North and Nobel 1998; Wang et al. 2005), although differences exist among plant species and cultivars in the ability to exploit such patches (Huang 1999; Blum and Johnson 1993; Gallardo et al. 1996). Drying roots signal to the shoots resulting in partial stomatal closure aiding plant water conservation (Croker et al. 1998; Davies et al. 2000; Sauter et al. 2001). The importance of roots in the signal pathway was demonstrated by the study of Gowing et al. (1990) using young apple (*Malus* x *domestica* Borkh) plants with their roots split between two tubes. The roots in one tube were well-watered, while those in the other tube were allowed to dry for 24 days, resulting in a reduction in leaf initiation and expansion compared to controls with their entire root system experiencing well-watered conditions. Excision of the roots in the tube experiencing the dry conditions resulted in recovery of leaf growth rate compared to plants that continued to have half of their root system under dry conditions. Though the study by Gowing et al. (1990) demonstrated the role of roots in signalling reduced water supply to the shoots, the exact nature of this signal is still a matter of considerable debate. While most studies have focused on either the involvement of chemical signalling involving abscisic acid (ABA) or hydraulic signals (reviewed by Comstock 2002; Ren et al. 2007; Neumann 2008), more recently it has been proposed multiple signals including xylem pH and hydraulic signals may interact with ABA to regulate the stomatal response (see Ren et al. 2007; Schachtman and Goodger 2008).

Plants can shift their water uptake away from dry areas to wetter areas with the well-watered side of the root system increasing its absorption capacity to compensate for the part of the root system experiencing dry conditions (Green and Clothier 1999; Moreshet et al. 1983; Liu et al. 2008). For example, kiwifruit vines (*Actinidia deliciosa*) rapidly shifted their water uptake from drier to wetter areas (Green and Clothier 1995). Following rewetting, roots in the dry zone rapidly recovered and reached a level of activity greater than it had been prior to drying. This increased activity was thought to be associated with new root production (Green and Clothier 1995). Similarly, potato (*Solanum tuberosum*) roots with part of their root system experiencing dry conditions showed an exponential decline in the fraction of soil water extracted with declining soil water potential, but following irrigation immediately recovered their capacity for water uptake (Liu et al. 2008). Supplying water

to only half of the root-zone of peach (*Prunus persia*) seedlings was sufficient to meet the complete water requirements of these seedlings (Tan and Buttery 1982).

3.1 Agricultural and Horticultural Applications

The exploitation of the ability of roots to cope with only part of the root system receiving water has considerable potential in horticulture and agriculture as a mean to conserve water by reduced irrigation and to modify the signalling and growth response within the plant (Davies et al. 2002). Partial root-zone drying (PRD) is one such reduced irrigation strategy where two halves of the root system are watered alternately. Switching the irrigation prevents roots drying out completely and dying, which would also result in loss of the root–shoot signal. Compared to other forms of irrigation, PRD irrigation of mature grapevines (*Vitis vinifera*) have been found to reduce vegetative growth, but not fruit yield and, in some cases, improved fruit quality (Dry and Loveys 1999; dos Santos et al. 2007; de Souza et al. 2005). Moreover, the results of some studies on potato suggest PRD does not result in significant yield reductions compared to that of fully irrigated, non-deficient, controls despite less water being supplied to the plants (Shahnazari et al. 2007; Saeed et al. 2008). In contrast, adding less water to only a fixed half of the root system of pear trees resulted in greater yields and increased irrigation water use efficiency (IWUE) than when both sides were alternatively watered (Kang et al. 2002). This may have been due to the "dried" pear roots still being able to extract water from a shallow ground water table (Kang et al. 2002), but as root distributions among the different treatments were not followed this is uncertain.

In the comparisons discussed above, the amount of water added differed among the treatments. Arguably, a more appropriate comparison of the potential of PRD is with treatments that supply the same amount of water but without switching (such as conventional deficit irrigation (CDI) treatments), although such a comparison is not always included (e.g. see studies by Shahnazari et al. 2007; Poni et al. 2007). In those studies which have drawn this comparison the benefits of PRD are often less pronounced. For example, in tomato plants, both fresh and dry mass of fruit per plant, fruit number and fruit water content on a dry mass basis were not different, if water was supplied alternatively to half the root system at a time or split equally between the two halves of the root system (Zegbe-Domínguez et al. 2003). Moreover, the mean fresh mass of fruit (i.e. fruit size) was actually significantly higher when both parts of the root system were watered equally compared to the PRD treatment, although maturity of the PRD fruit was advanced by a week, which may confer a commercial advantage (Zegbe-Domínguez et al. 2003). In field grown maize, yields and IWUE were not enhanced by PRD compared to a CDI treatment (Kirda et al. 2005).

PRD has, however, been suggested to have additional benefits, such as enhancing microbial activities, which in turn may benefit plant growth (Wang et al. 2008), improving nitrogen availability for the crop (Shahnazari et al. 2008) and reducing

nitrogen leaching (Kirda et al. 2005), although if these additional benefits are found under all soil types or conditions is currently unknown. Thus, the benefits of PRD over CDI are still a matter of debate and the conditions under which PRD studies have been conducted including the comparisons among other irrigation treatments does require careful consideration when trying to evaluate the benefit of PRD as an irrigation strategy. Moreover, the growth stage at which the irrigation treatments are applied, the duration of the treatment (including the frequency of switching watering between the two sides of the root system) and the crop in question are all likely to be critical to the response observed (e.g. Costa et al. 2007; de la Hera et al. 2007; Kirda et al. 2007).

4 Hydraulic Redistribution: How Roots Influence Water Distribution

Roots act as key conduits of water. Transpiration is the main driving force of water movement through the plant, however, during periods of low transpiration, such as night-time and other periods when partial stomata closure occurs, water can move from roots experiencing moist layers to those in drier areas. Some of this water subsequently leaks out through the roots and is recaptured by the same or neighbouring plant roots to meet the transpiration demands of the plant the next day (Ryel et al. 2002; Kurz-Besson et al. 2006). This passive movement of water was first reported for deeper roots experiencing wetter conditions moving water upwards to roots in the upper drier soil layers, and thus, was termed "hydraulic lift" (Richards and Caldwell 1987; Caldwell and Richards 1989). However, water movement downwards (Burgess et al. 1998; Schulze et al. 1998) and even horizontally (or transverse) (Smart et al. 2005; Burgess and Bleby 2006) has subsequently been reported. In fact, the movement of water through roots may occur in any direction, providing there is a sufficient soil water potential (Ψ_{soil}) gradient and that roots with hydraulically intact pathways are present (Leffler et al. 2005; Oliveria et al. 2005). Consequently, this process of water movement is now mainly referred to as "hydraulic redistribution" (Burgess et al. 1998).

4.1 Importance of Hydraulic Redistribution

Hydraulic redistribution has been reported in over 50 plant species from a range of habitats (Jackson et al. 2000; Lee et al. 2005) and allows both photosynthesis and evapotranspiration to increase (Lee et al. 2005), in addition to potentially having a large impact upon the hydrology of the landscape (Jackson et al. 2000; Scott et al. 2008). The size of this impact will depend upon the amount of water that is redistributed and used in the next day for transpiration. Reported values do vary considerably with differences among years (Muñoz et al. 2008), seasons (Scott et al. 2008), extent of shading (Williams et al. 1993), plant species present (Hultine et al.

2003; Dawson 1996) and the spatial distribution of roots and moisture in the soil (Warren et al. 2007), among the factors responsible for this variation. Kurz-Besson et al. (2006) estimated that hydraulically redistributed water contributed ca. 17–81% (with larger contributions from trees presenting the lowest xylem hydrogen isotopic signatures (δD_{xylem})) of the total water used for transpiration the following day by 15-year-old *Quercus suber* trees in a savannah-type Mediterranean ecosystem. For temperate forests, Dawson (1993) estimated the increase in transpiration as a result of hydraulic redistribution was ca. 19–40%, while using a modelling approach, Lee et al. (2005) estimated hydraulic redistribution increased transpiration over the dry season in the Amazon by ca. 40%. This movement of water over the dry season of the Amazon enables these forests to avoid water stress (Oliveria et al. 2005), and permits increased photosynthesis while enhancing latent cooling, thus lowering the surface-air temperatures (Lee et al. 2005). Thus, the process of hydraulic redistribution by roots also has important consequences for global climate change parameters.

4.2 Ecological Roles of Hydraulic Redistribution

Although the exact ecological roles of hydraulic redistribution are uncertain, it has been proposed that it aids not only the species conducting the redistribution, but also neighbouring plant root systems. The possible ecological impact of hydraulic redistribution has been the topic of several reviews (e.g. Caldwell et al. 1998; Horton and Hart 1998; Ryel 2004). Thus, here only the more recent literature will be examined in detail.

While the redistribution of water to drier areas in the soil profile is a passive process and not under plant control, it does confer a number of benefits to the plant including extended root longevity (Bauerle et al. 2008; Smart et al. 2005) and increased root membrane integrity (Bauerle et al. 2008) in drier areas. However, though hydraulic redistribution is a passive process, plant species do vary in their ability to perform hydraulic redistribution suggesting selection pressures have operated on various plant species to either promote or reduce water loss through this process. The release of water into drier layers of soil may also protect beneficial microorganisms, such as mycorrhizal and saprotrophic fungi against hyphal desiccation (Querejeta et al. 2007; Warren et al. 2008) allowing these fungi to maintain function including nutrient release for plant uptake. Although pathogenic fungi may also benefit from hydraulic redistribution, which could be detrimental to the plant and its neighbours, as the mycorrhizal fungi are in such close association with the plants root system they are ideally positioned to take advantage of any "root" released water before its release into the rhizosphere soil (see also Querejeta et al. 2003). In the natural environment, mycorrhizal hyphae may link different plants in the community together via a common mycorrhizal network (CMN), and there is some evidence that hydraulically distributed water may move via the CMN into other plant roots (Egerton-Warbuton et al. 2007; Plamboeck et al. 2007).

However, the relative importance of movement via the CMN directly versus uptake from soil pathways remains uncertain (see Schoonmaker et al. 2007).

Although most nutrient inputs to soil are in solid form, roots generally acquire nutrients from the soil solution phase. Moreover, as plants transpire, water and thus, nutrient ions contained within the soil solution move to the root via mass flow. Diffusion is the other main form in which nutrients move to roots. Water movement to drier soil layers via hydraulic redistribution may, therefore, be expected to enhance both these ion movement pathways, and so may potentially reduce the heterogeneous distribution of nutrient supply in these layers. However, the limited evidence available suggests hydraulic redistribution did not reduce nutrient heterogeneity (Caldwell and Manwaring 1994).

In most soils, the majority of nutrients are concentrated in the upper soil layers. Thus, hydraulic redistribution may only be expected to enhance nutrient capture when upward movement of water (i.e. "hydraulic lift") occurs. However, Rose et al. (2008) found hydraulic lift did not prevent a large reduction of phosphorus capture by two canola cultivars with the larger rooted cultivar being the most adversely affected. The reduction in nutrient acquisition was probably a result of minimal fine root growth following drying of the topsoil. In contrast, McCulley et al. (2004) demonstrated that if soils also contained large stores of available nutrients at depth, then deep roots did have the ability to acquire these nutrients via the redistribution of water downwards. Thus, even though absolute nutrient concentrations were higher in the upper soil layers, in this case deep roots also played an important role in nutrient capture aided by hydraulic redistribution. Snyder et al. (2008) tested if hydraulic redistribution or night-time transpiration, the incomplete closure of stomata during the night (Caird et al. 2007; Dawson et al. 2007), had the greater impact upon nitrate acquisition. Although all plants took up the added nitrate, the size of the hydraulic lift had no effect on the amount acquired. In contrast, plants that had night-time transpiration suppressed tended to have lower nitrate acquisition, although the effect was only weakly significant ($P = 0.07$). Thus, the impact of hydraulic redistribution on nutrient availability is still uncertain.

5 Roots and Nutrient Foraging

Nutrient availability in soil is heterogeneous both spatially and temporally (Stark 1994; Fitter et al. 2000). This variation in nutrient supply can be considerable. For example, using geostatistical analysis Jackson and Caldwell (1993a, 1993b) demonstrated, there was as much variation in nutrient availability within the rooting zone of an individual plant as within a 120 m^2 plot. While Farley and Fitter (1999a) found nitrate and ammonium concentrations in the soil solution from a deciduous woodland varied two- to fivefold at scales of only 20 cm. Moreover, the temporal pattern of nutrient availability was highly variable (Farley and Fitter 1999a). It is well established that plant roots can show considerable plasticity in both their morphology and physiology to enable them to cope with such nutrient

heterogeneity and exploit nutrient-rich zones or patches when they become available (Hodge 2004, 2006). Allocation of resources belowground is usually enhanced under low nutrient availability (Reynolds and D'Antonio 1996). In addition, more discrete changes in the root system may occur through changes in root system architecture (RSA) (Fitter 1994). Moreover, just as roots are able to change resource allocation to areas of the root system experiencing well-watered conditions compared to those in drier areas (see Sect. 3), roots can also allocate additional resources to nutrient-rich zones resulting in increased root growth in these areas often at the expense of growth in nutrient-poorer regions (Granato and Raper 1989; Drew 1975).

5.1 Differences Among Species in Biomass Allocation to Nutrient-Rich Zones

In an ingenious experiment conducted by Campbell et al. (1991), competitively dominant and inferior or subordinate plant species were screened for their ability to place roots in nutrient-rich zones. The plants were grown in microcosm units and on the surface of the growth medium, nutrient solution was dripped. By achieving the correct drip rate, Campbell et al. (1991) was able to present the roots with two nutrient-rich and two nutrient-poor quadrants without the use of barriers, thus allowing free root growth in the unit. In other studies, such as the classic work on root proliferation by Drew and co-workers in the 1970s (Drew 1975; Drew and Saker 1975, 1978), only part of the root system had been exposed to nutrient-rich conditions and free root growth between rich and poor areas had not been possible. The competitively dominant plants had the greatest amount of root in the nutrient-rich zones, whereas the subordinate species placed more of their new root growth in the nutrient-rich patches. Campbell et al. (1991) referred to this as a trade-off between the *scale* and *precision* of the response. The competitively dominant plants could respond by scale because they had absolutely more root, whereas the subordinate species, although they had fewer roots, placed their new root biomass with more precision in areas where they would derive most benefit.

The idea of a "scale-precision trade-off hypothesis" has intrigued root researchers ever since and has prompted much recent debate in the literature as to its importance and application (see Kembel and Cahill 2005; de Kroon and Mommer 2006; Grime 2007; Kembel et al. 2008). However, the hypothesis has been widely tested for a range of plant species from different environments and has received only limited support (Kembel and Cahill 2005; Bliss et al. 2002; Einsmann et al. 1999). In contrast to the original hypothesis, the results of some studies even suggest that precision of foraging is greater in species with larger root systems (Einsmann et al. 1999; Farley and Fitter 1999b). For example, Einsmann et al. (1999) found that when only herbaceous plants were compared, a strong, *positive* relationship between scale and precision was observed, although the sample size in this case was small ($n = 6$). However, in neither study (i.e. Campbell et al. 1991 or

Einsmann et al. 1999) did the plant species selected actually co-occur. In Campbell et al.'s (1991) case, species came from several communities and were selected because they were common to the UK, in the case of Einsmann et al. (1999), the species selected were in a successional sequence, thus did not actually occur together at the same time. In the study by Farley and Fitter (1999b), the plant species tested did co-occur, yet here also no evidence for a scale-precision trade-off was observed. In contrast, Johnson and Biondini (2001) screened 59 plant species from the Great Plains grassland, USA and found some limited support for the scale-precision hypothesis in that the species screened that showed a higher level of plasticity were subdominant species. However, Johnson and Biondini (2001) also concluded that small scale heterogeneity and root plasticity was not important for plant competition or nutrient acquisition as most species showed similar performance regardless of the extent of nutrient patchiness. Moreover, in a meta-analysis conducted by Kembel and Cahill (2005) of the scale-precision literature, no scale-precision trade-offs either within or between habitats was evident, including the study conducted by Johnson and Biondini (2001). Thus, there is currently little evidence that co-occurring competitively dominant and subordinate species adopt different strategies in terms of scale and precision and that these strategies promote species co-existence.

5.2 Root Demography

Measurements of root biomass alone (Sect. 5.1), only provide information on the amount of root biomass present at that particular sampling time and provide no information on the amount of root production and death that occurred to result in that biomass. To acquire this information, root demography measurements are required.

Fine roots do tend to turnover more rapidly and it is these roots that are primarily involved in nutrient acquisition and patch exploitation. However, what actually constitutes a "fine" root has received much debate in the literature and grouping roots together based on arbitrary class (diameter) sizes without considering their position on the root system, their depth in the soil and so forth, has been much criticised (Pregitzer 2002; Zobel 2003). Thus, determining root branch order may be a better approach than actual root diameter to assess the role of different fine root populations in soil (Guo et al. 2008). Using such an analysis, Guo et al. (2008) found that only a fraction of "fine" roots turned over annually and that neither N addition nor removal of foliage had a significant effect on root lifespan.

Although N fertilisation did not influence root lifespan in the study by Guo et al. (2008), fine roots have been shown to have increased N concentrations and respiration rates and there is a strong relationship between these two factors (Reich et al. 1998; Tjoelker et al. 2005; Pregitzer et al. 1998). In addition, root (and leaf) longevity is generally greater in nutrient-poor habitats compared to nutrient-rich (Aber et al. 1985; Pregitzer et al. 1995; Ryser 1996). Presumably, this helps to

reduce the cost (in terms of carbon (C) and nitrogen (N)) associated with new root construction. However, the plant may off-set such costs by withdrawing N from fine roots shortly after construction (Volder et al. 2005), and respiration rates (thus C costs) may also decline with age (Tjoelker et al. 2005; Bouma et al. 2001), but so too does nutrient acquisition (Bouma et al. 2001).

5.2.1 Root Demography in Nutrient-Rich Patches

Decreased root longevity might, therefore, be expected in nutrient-rich patches similar to nutrient-rich soils. However, as for changes in root demography as a result of water availability (see Sect. 2.2), no one strategy appears optimal for roots in nutrient-rich patches (see Table 1). An increase in root production generally occurs, but not always. For example, in the study by Hodge et al. (2000c), the rate of root production was slowest in the most complex organic patch added (*Lolium perenne* shoot material) and cumulative root births in this patch did not differ from those in the controls receiving only water. In contrast, root production accelerated when patches of low C:N ratio (hence more readily decomposed) were added. Root mortality, however, did not differ over the 48 days monitoring period (Table 1).

Differences among studies in the species and conditions used make comparisons more difficult as root demography is known to be influenced by a wide range of biotic and abiotic factors (see Hodge 2004; Eissenstat and Yanai 1997). The actual method employed to assess root demography also influences the results obtained (Hendricks et al. 2006; Pierret et al. 2005; Withington et al. 2003). Although Pärtel and Wilson (2001) observed both increased root production and mortality in fertiliser patches (Table 1), overall heterogeneous soils showed reduced root production and increased root longevity compared to that in the homogenous soil. Thus, *F. rubra* was able to alter its root demography within the heterogeneous treatment in response to the increased nutrients available in the fertiliser patch compared to elsewhere. Similarly, another grass species, *L. perenne*, altered its root demography in response to the size and concentration of the L-lysine patch, which its root system encountered (see Hodge et al. 1999a, Table 1).

An additional problem when trying to determine the benefits of contrasting demographic responses to nutrient patches is that some studies do not actually report what the differing effects of root demography actually resulted in terms of the amounts of nutrients acquired from the added patches. This hinders any cost-benefit analysis of the observed demographic response being determined. Obviously in field studies, such measurements are more problematic, but are certainly achievable in pot or microcosm studies. For example, although *L. perenne* grown in microcosms altered its root demography in response to L-lysine patches of varying size and concentration (Table 1), absolute N capture from the L-lysine was a simple function of the amount of N added. However, the *relative* N capture was patch dependent. Furthermore, despite the proliferation of roots in the highest N containing patch, only 29% of the available N was captured by the plants from this patch compared to 40–47% N captured from other treatments. This was

Table 1 Effect on root production, mortality and longevity in nutrient-rich patches from various studies

Species	Location	Nutrient treatment	Effect on root demography	Reference
Mixed hardwood forest, northern Michigan USA	Field study	Pulses of water only or water + N (as NH_4NO_3) applied over 20 or 40 days; twice the amount of H_2O + N or H_2O only was applied over 40 days cf 20 days	Increased root production and increased root longevity in both types of patches; water + N increased root production more than water alone and this production was higher in the 40 days treatment	Pregitzer et al. (1993)
Four old-field plant species (*Ambrosia artemisiifolia; Chenopodium album; Achillea millefolium* and *Bromus inermis*)	Controlled condition greenhouse; pot study	Injection of N, P, K fertiliser solution or water (control) to either side of the pot	For all species combined root numbers, root death and root length increased in the fertilised zones compared to water controls	Gross et al. (1993)
Festuca rubra	Controlled condition greenhouse; pot study	Nutrient-poor soil with N,P,K fertiliser spread uniformly (homogeneous treatment) or concentrated to one-third of the pot side (heterogeneous treatment)	Increased root production and increased mortality in fertiliser patches. No difference in root longevity	Pärtel and Wilson (2001)
Lolium perenne sward	Greenhouse; microcosm study	L-lysine added as a single addition ("patch") or as a series of smaller pulses at 7 day intervals over 28 days. Controls as above except a patch or pulse of water added	Instantaneous root birth rate greater in L-lysine pulse at 21 days only. Cumulative births in L-lysine patch greater than water patch in the later experimental phase (25–48 days). Largest effect on root deaths which were faster in the L-lysine treatments regardless of if a patch or pulse applied	Hodge et al. (1999c)
Lolium perenne sward	Greenhouse; microcosm study	L-Lysine (a simple amino acid) injected as a solution of various size and concentration to part of the root system	Various effects on root demography. In the most enriched patch root production and death increased. In the	Hodge et al. (1999a)

			weakest patch initially root birth rate increased and no change in death rate. In three treatments that supplied the same amount of N the two with the lowest concentration showed no change in either root birth or death rate while in the most concentrated of the three root death rate decreased but birth rate was unchanged	
Lolium perenne sward	Greenhouse; microcosm study	Organic patches of varying chemical and physical complexity added. Patches with a low C:N ratio were algal lyophilized cells, algal amino acid mixture, L-lysine and urea. *L. perenne* shoot material was added as a patch of a high C:N ratio. Controls received water only	Root production increased in patches with a low C:N ratio compared to that with the highest C:N ratio (i.e. *L. perenne* shoots). No difference in root mortality over a 48 days viewing period	Hodge et al. (2000c)

probably due to intense plant–microbial competition in the highest N patch (Hodge et al. 1999a, 2000a).

5.2.2 Root Demography and Microbial Responses to Nutrient-Rich Patches

Microbial breakdown of organic nutrient patches is important for the subsequent release of nutrients for plant capture. Plants may also be able to acquire N directly from organic compounds (Chapin et al. 1993; van Breeman 2002), although the ecological significance of this N pathway is still a matter of some debate (Jones et al. 2005). Moreover, if microorganisms or plant roots are the superior competitors for both inorganic and organic N sources continues to be the focus of much attention in the literature (see reviews by Hodge et al. 2000a; Kaye and Hart 1997; Lipson and Näsholm 2001). To address this question, both the responses of plant roots and soil microorganisms to N sources in soil have to be followed through time. In a study by Hodge et al. ((1999c); see Table 1), the impact upon root demography and plant N capture of a *L. perenne* sward, following addition of ^{13}C:^{15}N dual labelled L-lysine either as a single large patch or as a series of smaller pulses at weekly intervals was determined. Surprisingly, neither the total nor the rate of N capture from the L-lysine additions differed between the patch or pulse treatments, which may reflect the lack of any marked root proliferation response between these two treatments (see Hodge et al. 1999c, Table 1). In contrast, the amount of ^{13}C (as $^{13}CO_2$) recovered from the decomposing L-lysine and detected by periodic sampling of the soil gas was generally higher from the pulse treatment (Fig. 1). Thus, although the roots did not generally respond to regular additions of a small L-lysine pulse, as shown by no difference in the rate of N capture in regular shoot sampling compared to the L-lysine patch treatment, the microorganisms did, particularly when plant roots were also present (Fig. 1). Given the large volume of literature that has been published on root responses to nutrient heterogeneity, it is surprising that so little attention has been paid to the interaction between soil microorganisms, roots and patches. Protozoa and nematode numbers, a surrogate for microbial activity, were found to rapidly increase in organic patches of dead earthworm and *L. perenne* shoots. In contrast, plant N uptake from both patch types in the early experimental phase was low, but increased later as the populations of protozoa and nematodes declined (Hodge et al. 2000d), suggesting plant roots capture larger amounts of N from patches in the longer term as they represent a slower turnover pool (see Hodge et al. 2000a).

Any changes in root demography would also be expected to impact on the soil microbial community through enhanced rhizodeposition as a result of increased root production, leading to greater exudation, or increased root death. When nitrate was supplied to only one side of *Hordeum vulgare* (barley) plants with their roots split between two containers, the roots receiving the nitrate did show enhanced release of recent photoassimilate into the rhizosphere (Paterson et al. 2006). If overall root system exudation in a heterogeneous compared to a homogeneous

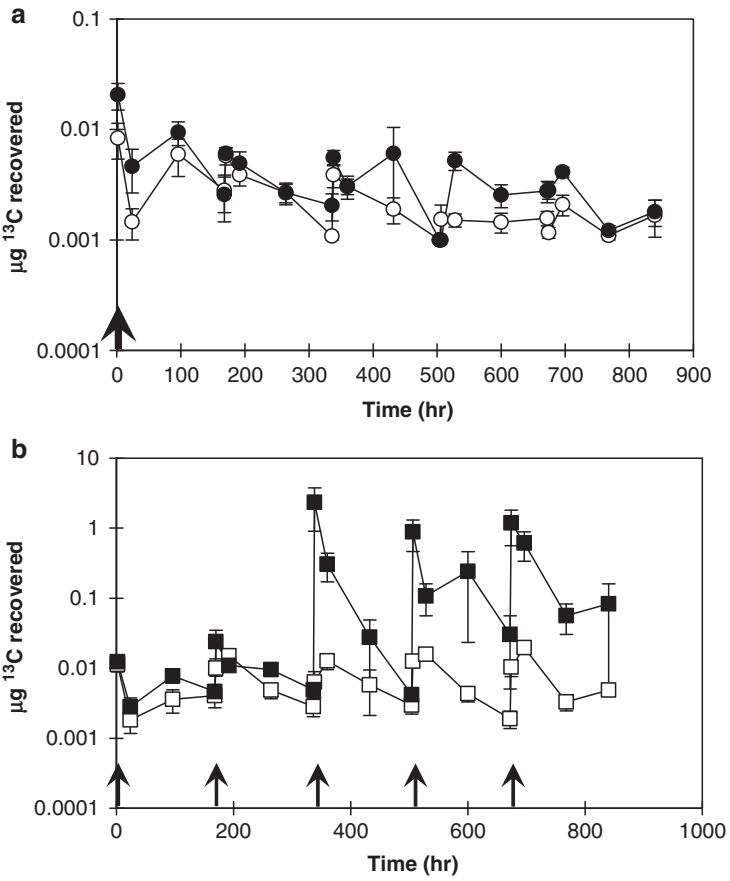

Fig. 1 Amount of ^{13}C (as $^{13}CO_2$) recovered over time from periodically sampling the soil gas atmosphere from tubes containing the (**a**) L-lysine planted (*filled circle*) and unplanted (*open circle*) patch treatments and (**b**) L-lysine planted (*filled square*) and unplanted (*open square*) pulse treatments. Data are means ($n = 6$) with SE bars. In the patch treatment, all the L-lysine was added at the start, whereas in the pulse treatment, a smaller quantity was added with time. *Arrows* indicate the time of L-lysine patch or pulse additions. Note the difference in scale on graph (**b**). Reprinted from Hodge et al. (1999c). Copyright (1999), with permission from Elsevier

nutrient environment differed, however, was not determined. Recent evidence suggests that as yet unidentified compounds released from roots inhibit growth (Falik et al. 2005; Semchenko et al. 2007). If these compounds similarly accumulate in nutrient-rich patches, root growth would be expected to be regulated. However, an increase in both root numbers and length are often reported in nutrient-rich patches (Pregitzer et al. 1993; Hodge 2004), suggesting that these inhibitory compounds diffuse rapidly into the surrounding soil preventing a build-up to inhibitory levels, or that they are consumed by the increased microbial

biomass in the patch zone. The studies on these inhibitory compounds have been conducted in largely artificial medium, thus their importance in the complex soil environment has yet to be determined.

5.3 Other Root Responses to Nutrient Heterogeneity

Changes in the architecture of the root system (i.e. its spatial configuration) can occur in response to nutrient heterogeneity (Fitter 1994) and a wide range of other environmental conditions (Malamy 2005). Changes in RSA depend on the nutrient available and much work has focused upon differences in RSA as a result of nitrate or phosphate availability (reviewed by Desnos 2008). Moreover, the root cap likely plays a key role in detecting nutrient deficiency, which subsequently results in arrested root growth on low phosphate medium (Svistoonoff et al. 2007). As such, RSA is currently the focus of much interest for crop improvement to enhance yields under current global change scenarios including decreased water availability as well as enhanced nutrient capture. Consequently, RSA has been the topic of many recent reviews covering both the genetic controls and potential for genetic improvement (de Dorlodot et al. 2007; Hardtke 2006), in addition to RSA responses to nutrient availability (Desnos 2008; Osmont et al. 2007; Malamy 2005).

Most work on RSA has focussed on model plants, such as *Arabidopsis*, for obvious reasons due to the enhanced genetic information available and the readily supply of mutants. On other plant species with more extensive root systems, detailed RSA measurements can be quite challenging and time consuming. Consequently, changes in root length in nutrient-rich patches are more frequently reported. However, length may increase through greater elongation of existing roots as well as through new root initiation and it is the latter that really ought to be used to demonstrate root proliferation *per se* (Hodge 2004). Root length is a better measurement than biomass as it provides more information on the absorptive capability of the root system than biomass alone. In addition, SRL (i.e. length of root per unit dry mass; cm mg^{-1}; SRL) has also been shown to correspond with the rate of root proliferation in nutrient-rich zones (Eissenstat 1991). However, SRL is not always a good predicator of root proliferation (Hodge et al. 1998) and it is itself plastic in that SRL can alter in nutrient-rich zones compared to nutrient-poor areas due to a greater length of thinner root. Moreover, SRL may be similar among contrasting species, yet actual tissue density may differ, which in turn may be an important predictor of root longevity (Ryser 1996, 2006).

Changes in ion uptake or physiological plasticity, although less often measured can also increase in nutrient-rich patches (Jackson et al. 1990; van Vuuren et al. 1996; Wang et al. 2006) and can be more important for nutrient acquisition than actual root proliferation responses (van Vuuren et al. 1996), although this may vary with plant species (Wang et al. 2006). Again, there are exceptions with

morphological plasticity demonstrably more important for nutrient capture in some studies (Kume et al. 2006; Hodge et al. 1999b). It is likely that the predominance of a morphological or physiological response is context dependent and can vary within plant species among specific habitats depending on prevailing abiotic conditions (Derner and Briske 1999; Hodge 2004).

5.4 Mycorrhiza Responses to Nutrient Heterogeneity

Although it is well established that mycorrhizal associations between the majority of plant species and certain soil fungi confer nutritional benefits to the associated host plant (Smith and Read 2008), much less is known about how these fungi aid nutrient capture specifically from patches. Here, only the arbuscular mycorrhizal symbiosis (AM) will be considered as it is the association on which most studies on patches have been performed. The AM association is the most common mycorrhizal symbiosis and can form between ca. two-thirds of all land species and fungi within the phylum Glomeromycota. AM fungi, unlike those fungi involved in either the ericoid or ectomycorrhizal associations, are not believed to have any saprophytic capability. This has traditionally led to these fungi being viewed as mere extensions of the plant root system despite these fungi being organisms in their own right with their own nutritional demands.

The results of the few studies that have examined the impact of AM colonisation when both the fungus and the colonised roots are present together in the patch suggest that the fungal symbiont does not confer additional benefits in terms of N capture to its associated host in comparison to non-mycorrhizal controls (Cui and Caldwell 1996a; Hodge 2001, 2003a; Hodge et al. 2000b). A notable exception occurred when two mycorrhizal plant species were in inter-specific plant competition, when being mycorrhizal, did enhance N capture from an organic source (Hodge 2003b). The N captured from the organic material was dependent on the root length produced, which was higher when the AM fungus was present. AM fungi have also been observed to enhance root proliferation within an organic patch (Hodge et al. 2000b). Thus, when both roots and AM fungi are present in an organic patch, the response by the roots appear more important for N capture, although AM fungi may modify this response. In addition, there is some evidence when both roots and the fungi have access to patches, the fungus may enhance P acquisition (Cui and Caldwell 1996a), although only one plant species out of seven tested showed such enhanced P acquisition in the study by Farley and Fitter (1999b).

In contrast, when only AM fungi and not roots have access to nutrient patches, then the AM fungus has been found to transfer both N (Hodge et al. 2001; Leigh et al. 2009) and P (Cavagnaro et al. 2005; Cui and Caldwell 1996b) from the patch to its associated host plant. Different species of AM fungi, however, vary considerably in their ability to transfer these nutrients to their associated host (Leigh et al. 2009; Cavagnaro et al. 2005).

6 Root System Trade-offs: Coping with More Than One Environmental Variable

Roots have to cope with multiple stresses in the soil environment, yet only a small number of the genes expressed to different stresses may actually be shared. For example, Dinneny et al. (2008) found that osmotic stress, caused by a high salt medium, and nutrient stress due to growth on an iron-deficient medium, shared only ca. 20% of regulated genes yet both stresses would be expected to impact on root growth. However, as stated previously (see Sect. 2.1), ecological evidence suggests water and nutrient uptake may differ along the root system and competition for water and N may also differ. Thus, this may explain the lack of similarity in gene regulation between these two stresses. If different nutrient deficiencies, which have identical impacts on root growth only share a small number of regulated genes remains to be determined.

Jansen et al. (2005) tested four flood-sensitive and four flood-tolerant species for their ability to selectively place roots in nutrient-rich patches. Although flood tolerance and nutrient heterogeneity were tested in separate experiments, there was a negative correlation between these two parameters when seven out of the eight species were included in the analysis. The eighth species, *Rumex palustris*, was unusual in that it showed both high flood tolerance and selective root placement in nutrient-rich patches. These results suggest that, at least in some species, there is a root system trade-off between the ability to respond to flooding and nutrient heterogeneity. In contrast, three floodplain tree species differing in their waterlogging tolerance all showed preferential root proliferation in nutrient-rich areas under uniformly drained or waterlogged conditions (Neatrour et al. 2007). However, when both waterlogging and nutrient distributions were heterogeneously supplied together (i.e. in the same half of the pot), the least tolerant of the three species (i.e. *Liquidambar styraciflua*) avoided the waterlogged side at the expense of nutrient foraging. This did not, however, significantly affect the overall biomass presumably because those few roots that were present in the waterlogged-nutrient side were able to take advantage of the increased nutrient availability under waterlogged conditions. In contrast, the other two species tested (i.e. *Nyssa aquatica* and *Fraxinus pennsylvanica*) continued to forage effectively for nutrients when patches of nutrients and waterlogging coincided. When nutrient and waterlogging patches were spatially separate (i.e. when nutrients were concentrated in the drained side of the pot), however, both these species did not selectively place their roots in the nutrient-rich half of the microcosm unit, although the variability in allocation of roots among replicates was very high ranging from 93% of fine root length in the nutrient-rich half of the pot to 99% of root length in the waterlogged half (Neatrour et al. 2007).

In some studies, the response by roots to nutrient availability is greater than that of water (Coleman 2007; Pregitzer et al. 1993), while in others, root distributions are more closely related to soil water contents (Zhou and Shangguan 2007). Under phosphorus stress, shallow rooted bean genotypes grew best, while under

drought-stress, deep rooted genotypes did better. When both these stresses were combined, those genotypes with a dimorphic root system were the most effective (Ho et al. 2005). Wang et al. (2007) examined the effects of heterogeneous fertiliser placement and various water treatments on nutrient acquisition and biomass production by *Brassica napus* (oilseed rape). A fixed watering treatment to one quadrant of the pot resulted in strong root placement in that quadrant, which was not further enhanced by heterogeneous fertiliser placement into that quadrant. In addition, alternating drying and wetting of the soil did not affect the root placement response to the patches of fertiliser. Wang et al. (2007) concluded that the response of roots to water and nutrients may be independent of each other (see Sect. 2.1), while the results of Neatrour et al. (2007) suggest interactions may occur between water and nutrient capture under certain conditions. It is probably when exposed to multiple stresses, root system trade-offs occur (see Jansen et al. 2005; Ho et al. 2005), which in turn limit the extent of phenotypic plasticity that occurs in the natural environment (Valladares et al. 2007).

7 Conclusions

Plant root systems have evolved strategies to enable nutrient acquisition under different soils with contrasting nutrient deficiencies (Lambers et al. 2008) and water capture under different climatic regions (Sect. 2.1; Schenk and Jackson 2005). At a more local level, root systems can show both large and rapid changes in physiological ion uptake rates (Jackson et al. 1990) and extensive root proliferation in water (Sect. 3) and nutrient-rich (Sect. 5) patches. The fact that sometimes a physiological and at other times, a morphological response is shown to be the more important in nutrient acquisition implies that plants retain the capacity for both and that neither are redundant. It may be, as suggested by the recent findings of Poot and Lambers (2008), that too high specialisation of the plants root system may limit its competitivenesss in other environments and may also render the plant at a disadvantage when exposed to multiple stresses (see Sect. 6). Despite this, the majority of plant species tested demonstrate remarkable plasticity under controlled conditions often to a single limiting factor (e.g. Drew 1975). Thus, while the capacity for root system adjustment to water or nutrient limitation is large, its actual expression in the natural environment is often much more subtle. This is presumably due to the wide range of factors that all simultaneously impact upon the root system in the natural environment, which may increase the threshold before the root responds. Moreover, there are both costs and so limits to plasticity (DeWitt et al. 1998). Although costs can often be hard to fully evaluate, the increased fine root production in water and nutrient patches seemed apparently expensive given these fine roots often have high N concentrations. Furthermore, if the patch was short-lived the construction costs may outweigh the benefits. However, here too, recent evidence indicates the response is complex with N concentrations and respiration levels being drastically reduced shortly after construction, thus lowering overall

costs. This highlights the need to view roots not as a "static" root mass but as a dynamic system. Thus, while genetic and proteomic studies are underway to unravel the controls on root responses to water and nutrients (e.g. Zhu et al. 2007; Spollen et al. 2008; de Dorlodot et al. 2007), the role for ecologists is also of great importance in understanding the root response in the ecological context in which it is expressed, the impacts on other members of the plant community and overall nutrient dynamics in the soil–plant system.

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Re-Evaluation of Allometry: State-of-the-Art and Perspective Regarding Individuals and Stands of Woody Plants

Hans Pretzsch

Contents

Abstract Allometry, in its broader sense, is concerned with the size of organisms and its consequences for their shape and functioning. Since the postulation of the allometric equation in the 1930s, allometry, in a narrow sense, refers to analysis

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and modelling of logarithmic transformed bivariate size data by linear regression techniques.

This chapter first points out that allometric research built up a valuable set of hypotheses and biometrical methods for analysing size of organisms and its consequences for their shape and functioning. Then, a summary of the knowledge about allometry of woody plants and populations will unmask the search for overarching general allometric exponents of shape and form development largely as a hunt for a phantom. Tree size development and self-thinning processes in forest stands give evidence that allometric exponents certainly lie in a narrow corridor, but are species specific and superimposed by site conditions, mechanical disturbances, competition, and other types of stress. The discussion states that as long as allometry searches for universal constants to a certain extent, it stills our innate desire to reduce complexity and generalise. However, time is ready to focus on and understand the differences between the species, sites etc. in order to contribute to a better system of understanding. It is concluded that allometry has to draw attention both to the internal size-driven allometric partitioning process and to the external factors, which determine optimal biomass allocation. And at best, allometric research should analyse both factors in order to understand and integrate them. A systematic analysis, ordering and causal explanation of allometric exponents, which reflects an individuals' tricks and traits of optimising fitness, may provide an important link between plant genetics, physiology, plant biology and population biology. In contrast, application of inaccurate and imprecise general scaling rules can cause considerable flaws in modelling, prognosis and ecosystem management.

1 Introduction

1.1 Concept of Allometry and Biometrical Formulation

Allometry is a field of science concerned with the size of organs, organisms and populations and its consequences for their shape and functioning. As size and size relations in and between organisms reflect the result of the phylo- and ontogenetic evolution towards a functional optimisation (Niklas 1994, p. 1), the study of allometry seeks to understand the adaptations of living organisms to their environment.

1.2 Allometry in Its Broader Sense

A plant is a means and result of photoproduction at the same time. Its current size (e.g. the proportions between root and shoot) determines its access to resources and supply of building material. The latter is allocated and used for growth in

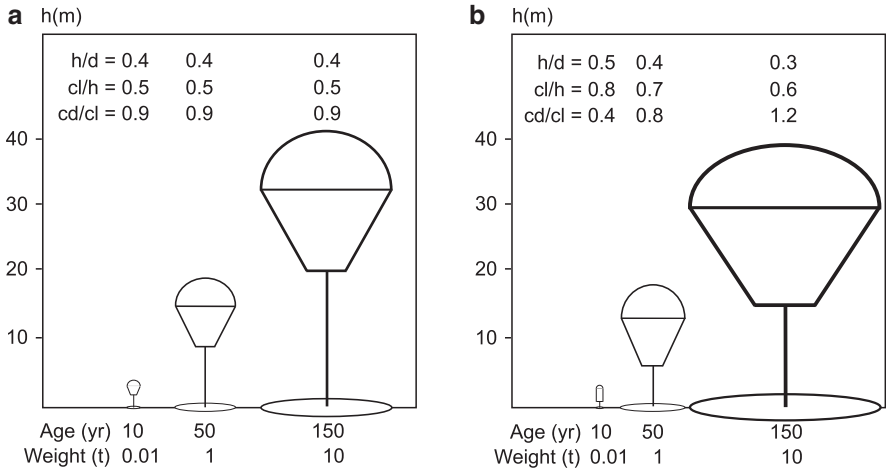

Fig. 1 European beech crown diameter-crown length development (**a**) with isometry, (**b**) with positive allometry, from age 10–150 in schematic representation (**a**) The tree represents geometrical similitude if all linear dimensions change proportionally to each other (e.g. diameter (cd) and length of the crown (cl) $cd \propto cl^{\alpha}$, $\alpha = 1$) and in general between all linear (lin), quadratic (quad) and cubic dimensions (cub) (e.g. tree height $\equiv lin$, crown projection area $\equiv quad$, tree biomass $\equiv cubic$) applies $lin \propto cub^{1/3}$, $lin \propto quad^{1/2}$, and $quad \propto cub^{2/3}$. (**b**) Mostly the relationships between tree dimensions change not proportionally but allometric with e.g. $cl \propto cd^{\alpha_{cl,cd}}$, $\alpha_{cl,cd} \neq 1$. In case of this European beech tree crown diameter (cd) and crown length (cl) are related like $cd \propto cl^{\alpha_{cl,cd}}$, $\alpha_{cd,cl} = 1.2$)

such a way that the altered plant size again fulfils the plant's functions (growth, reproduction) in an optimal way. Proportional size change (isometry) is mostly not adequate for optimal functioning. Rather, due to their specific efficiencies, organ size has to be changed unproportionally (allometry) for ensuring optimal function and supply of the plant with building material. That is the reason why with increasing size a tree's shape changes seldom proportionally, like the European beech shown on Fig. 1a. On the contrary, slender juvenile European beech trees have a rather small cd/cl-ratio between crown diameter cd and crown length cl, but with tree size width and cd/cl ratio of the crown increases as the crown diameter expands over-proportional in relation to tree height and crown length (Fig. 1b). Different phylogenetic pathways of functional optimisation lead to characteristic allometric developments of species. For example, an open grown European beech can be easily distinguished against an open grown Common oak by its shape – even from far distance. The science of allometry and the allometric biomass partitioning theory (APT) investigate the plants' internal feedback between size, shape and functioning (broken line in Fig. 2) and strive for general species-overarching rules for allometric partitioning and biomass allocation.

However, a plant's genetically determined shape (genotype) is not fixed as strictly as in animals but allows a comparably large ontogenetical plasticity (phenotype). For example, a European beech tree that grows in a forest environment

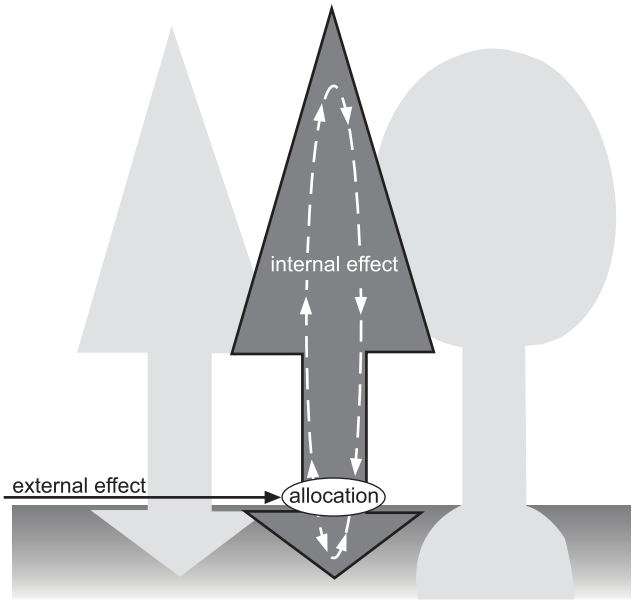

Fig. 2 Allometric research has to draw attention to both, to the internal size-driven allometric partitioning process (*broken arrow*) and to the external factors which determine optimal biomass allocation (*continuous arrow*). Competition for contested resources and externally driven disturbances like organ losses superimpose the internal size dependent allometric growth

differs substantially from the unhindered open-grown tree. Growing in a stand, a tree maximises its fitness by appropriating contested resources and adapting to the environmental conditions created by the neighbouring trees. In contrast to open-grown conditions, the tree's unhindered species-specific ontogenetic form development is superimposed by competition. According to the optimal biomass partitioning theory (OPT), light-limited trees in the understory, for example, boost their crown growth in order to improve the supply with the limiting light factor (van Hees and Clerkx 2003; Hofman and Ammer 2008). By contrast, in case of water or nutrient limitation, trees (regardless whether growing solitary or in a forest) enhance their root growth to overcome their limited access to these belowground resources (Comeau and Kimmins 1989; Kimmins 1997). In other words, external factors like a plant's competitive status within the stand, the site conditions, and the occurrence of disturbances (e.g. mechanical abrasion, crown breakage) affect the plant's supply with carbon, light, water and mineral nutrient. So, for a suppressed European beech in a dense mixed stand, species-specific allometry or generalised "spinach–redwood-allometric" relationship might apply in theory, however, competition and selective pressure modify allocation and shape to such an extent, that this tree hardly resembles an open grown or dominant beech. External factors (solid arrow in Fig. 2) continuously distract and superimpose the unhindered, primarily size driven plant allometry. So, allometry has to focus on both, the

internal size-driven allometric partitioning process and the external factors, which determine optimal biomass allocation; and at best, allometric research should analyse both factors in order to understand and integrate them.

In contrast to this wide approach to allometry as the study of relative size growth, which dates back to Thompson (1917) and is an issue again at present (e.g. McCarthy and Enquist 2007), allometry in a closer sense refers to the analysis and modelling of bivariate size relations of organisms, populations or communities by double-logarithmic relationships, which are introduced in the sequel.

1.3 Biometrical Analysis and Modelling of Plant Allometry

In the early 1930s, Huxley (1932) and Teissier (1934) formulated a "relative growth equation" that is today widely accepted as the allometric equation. Supposing x and y quantify the size of plant organs or a total plant, the growth x' ($\mathrm{d}x/\mathrm{d}t$) and y' ($\mathrm{d}y/\mathrm{d}t$) is related to the size x and y as

$$y'/y = \alpha\, x'/x. \tag{1}$$

Better known are the integrated and logarithmic representations given below.

$$y = ax^{\alpha}, \tag{1a}$$

$$\ln\ y = \ln a + \alpha \ln x. \tag{1b}$$

Allometry is the relative change of one plant dimension, $\mathrm{d}y/y$ (e.g. the relative height growth) in relation to the relative change of a second plant dimension $\mathrm{d}x/x$ (e.g. the relative diameter growth). Suppose the relationship between the two plant dimensions follows $y = a\,x^{\alpha}$, then $\mathrm{d}y/\mathrm{d}x = \alpha(1/x)ax^{\alpha}$ or $\mathrm{d}y = \alpha(1/x)ax^{\alpha}\mathrm{d}x$, so that

$$\mathrm{d}y/y/\mathrm{d}x/x = \frac{\alpha(1/x)ax^{\alpha}\mathrm{d}x}{ax^{\alpha}} \Big/ \mathrm{d}x/x = \alpha. \tag{2}$$

This shows, that the rate between the relative changes of the plant dimensions y and x is constant and equal to α, which is the allometric exponent in (1a). The allometric exponent α can be understood as a distribution coefficient for the growth resources between organs y and x: when x increases by 1%, y increases by α%. An individual tree height–diameter-allometry of $\alpha_{h,d} = 0.6$ means that height increases by 0.6% when tree diameter increase by 1%. Applied on stand level, a stem number–mean tree diameter-allometry of $\beta_{N,d\mathrm{q}} = -1.6$ means, that the stem number per ha decreases by -1.6% when mean tree diameter d_{q} increases by 1%. The exponents α and β, respectively, describe the slope when we plot $\ln(h)$ versus $\ln(d)$, respectively $\ln(N)$ versus $\ln(d_{\mathrm{q}})$.

The value pairs y_i, y_{i+1} and x_i, x_{i+1} from consecutive surveys of the plots can be used to calculate the period-wise slope $\alpha'_{y,x}$. It represents the distribution key of matter allocation between y and x in a given period.

$$\alpha'_{y,x} = \frac{\ln(y_{i+1}) - \ln(y_i)}{\ln(x_{i+1}) - \ln(x_i)} = \frac{\ln(y_{i+1}/y_i)}{\ln(x_{i+1}/x_i)}. \tag{3}$$

For infinitely small time steps, (3) corresponds to the quotient $\mathrm{d}y/y/\mathrm{d}x/x = \alpha$ (cf. (2)). In this chapter, allometric factors (a, b, ...) are distinguished from allometric exponents (α, β, ...) by Latin respective Greek letters (cf. e.g. (1b)). By writing α or β prime (e.g. α'), it is emphasised that the allometric exponent is based on short-term consecutive surveys (3); the subscripts of the allometric exponent (e.g. $\alpha_{y,x}$) show which size variables are addressed.

Narrowly defined, allometry refers to a set of methods for analysing bivariate datasets by applying the allometric equations (1)–(3) starting with logarithmic transformation of the size variables, application of regression analysis (e.g. OLS, RMA, PCA regression) to the ln–ln-transformed data, estimation of allometric factors and exponents, and finally the application of the obtained regression line to eliminate size-effects, to reveal species-specific or site-specific allometric exponents, and finally to interpret deviations from the fitted regression line as variability or plasticity.

1.4 Geometrical and Fractal Scaling

Because of the physiological significance of allometric exponents α (1a), they have been strongly discussed ever since. Since its beginning, allometric research was mainly driven by the search for an overarching, universal, allometric exponent. Often, it was proposed that volume or mass related allometric functions scale with exponents of 1/3 due to the volume dimensionality (von Bertalanffy 1951; Yoda et al. 1963, 1965; Gorham 1979). More recently, West et al. (1997, 1999), Enquist et al. (1998, 1999) and Enquist and Niklas (2001) presented a model for a general explanation of allometric scaling with exponents of 1/4, based on fractal networks of transportation systems in organisms (West-Brown-Enquist-model, short WBE-model).

Mostly, allometric analyses assume geometrical similitude as a starting point and null hypothesis. Geometrical scaling or similitude means that between different linear dimensions $\mathrm{lin}_1, \mathrm{lin}_2 \ldots \mathrm{lin}_n$ (e.g. tree height, diameter, crown length, height to crown base) applies proportionality $\mathrm{lin}_1 \propto \mathrm{lin}_2 \cdots \propto \mathrm{lin}_n$ (Fig. 1a). It further assumes that between quadratic tree attributes (e.g. basal area, leaf area, crown surface area, growing area) and linear dimensions applies $\mathrm{quad} \propto \mathrm{lin}^2$, and further between cubic variables like volume v or weight w (e.g. stem volume, crown volume, tree biomass) and linear dimensions applies $\mathrm{cub} \propto \mathrm{lin}^3$. That means $\mathrm{lin} \propto \mathrm{cub}^{1/3}$and

$$\mathrm{quad} \propto \mathrm{cub}^{2/3} \tag{4}$$

This corresponds to the 1/3 exponent scaling as three is in the denominator of the allometric exponent. Application of (4) to the relationship between mean tree volume $\bar{v}$ ($\equiv$ cubic) and mean growing area $\bar{s}$ ($\equiv$ quad) yields $\bar{s} \propto \bar{v}^{2/3}$. As $N \propto \bar{s}^{-1}$, we get the $-3/2$-power rule of self-thinning $\bar{v}^{-2/3} \propto N$ or $\bar{v} \propto N^{-3/2}$, which obviously assumes geometrical scaling and isometric form development (Yoda et al. 1963, 1965; Gorham 1979). Note that the $-3/2$ power rule belongs to the 1/3 exponent scaling, as volume scales to N with the power of $-2/3$. It has only become famous in the reverse formulation, where N scales to volume with the power of $-3/2$.

Fractal scaling in contrast assumes cub $\propto$ lin$^{\alpha}$ with $\alpha \neq 3$ and a deviation from 1/3 exponent scaling. For the relationship between weight (w) and tree stem diameter (d), Enquist et al. (1998, 1999) postulate $w \propto d^{8/3}$ with $ba \propto d^2$, the basal area–weight relationship results in

$$ba \propto w^{3/4}. \tag{5}$$

Obviously, fractal scaling leads to a quarter power (1/4-exponent) scaling rather than 1/3 exponent geometrical scaling. Applied on mean tree level, Enquist et al. (1998) derive $\bar{v} \propto N^{-4/3}$, which means a more shallow self-thinning line compared to $\bar{v} \propto N^{-3/2}$ from geometrical scaling. Enquist et al. (1998) stress that their model $\bar{v} \propto N^{-4/3}$does not predict self-thinning trajectories, but they do not explain why. This restraint makes their model's predictions somehow immune against falsification (Pretzsch 2006).

Note that when a statistical analysis of empirical data yields, e.g. $\bar{w}^{-2/3} \propto N <=> \bar{w} \propto N^{-3/2}$that implies not necessarily geometric scaling, e.g. a combination of fractal scaling $\bar{w} \propto \bar{d}^{8/3}$ and $\bar{s} \propto \bar{d}^{16/9}$would also yield $\bar{w} \propto \bar{s}^{3/2}$ or $\bar{w} \propto N^{-3/2}$ (as $N \propto \bar{s}^{-1}$). Specific deviations from geometrical scaling can obviously cancel each other so that an integrated view just looks like geometrical scaling, but is fractal in fact.

2 Formation of Shape and Form: Allometry on Plant Level

2.1 Above and Below Ground Allometry

The relationship between stem diameter and crown width is a good example of allometry relevant to the silvicultural management of pure and mixed stands of Norway spruce and European beech. Figure 3 displays the different space sequestration strategies of both species (crown width$\propto$stem diameter$^{\alpha}$) analysed for the unthinned mixed species stands, Freising 813/1-6 in South Bavaria (Pretzsch and Schütze 2008). Crown width, and hence, also the growing space requirement, increases with increasing stem diameter. Norway spruce exhibits an allometric exponent of $\alpha = 0.49$, European beech a significantly higher allometry of $\alpha = 0.60$. Consequently, for the same increase in diameter, European beech's demand

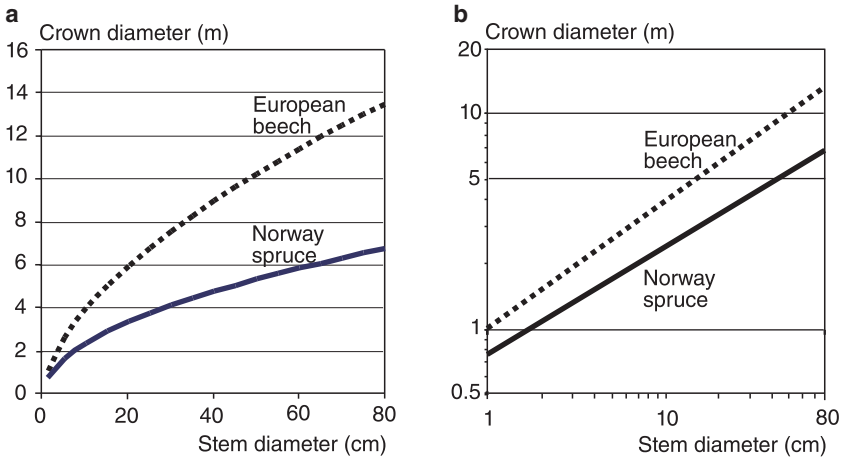

Fig. 3 Allometric relation between tree diameter (d) and crown diameter (cd) of Norway spruce (*continuous line*) and European beech (*broken line*) at long-term experimental plots Freising 813/1-6 in (**a**) Cartesian coordinate and (**b**) double-logarithmic system. The $\alpha_{cd,d}$-allometry shows the stronger crown expansion and steeper slope α of European beech ($\alpha_{cd,d} = 0.60$) compared with Norway spruce ($\alpha_{cd,d} = 0.49$)

for growing space is higher than that of Norway spruce. This species-specific allometry and growing space requirement long time is worked into the yield tables. For instance, the Norway spruce tables from Assmann and Franz (1965, cf. dominant height 40 m at age 100) and Schober's (1972) European beech tables (cf. yield class 1.0, moderate thinning) show that, at a mean tree diameter of 10 cm, there are expected some 3,500 trees ha^{-1} in a Norway spruce and 2,500 in an European beech stand (ratio 1.4:1). However, at a mean tree diameter of 50 cm, Norway spruce still has about 350 trees ha^{-1}, whereas there are less than 175 trees ha^{-1} (ratio 2:1) in the European beech stands due to its expanding crown allometry. This example has been selected to show the typically more lateral crown spread of broadleaves in comparison with the more vertical and pyramidal growth of the conifers (Niklas 1994, pp. 173–174). The species-specific differences in space requirement on individual tree level will reappear on stand level in species-specific slopes of the self-thinning lines (cf. Sect. 3).

The root–shoot development is maybe the best-analysed allometric relationship on plant level (e.g. Comeau and Kimmins 1989; Kimmins 1997; Shipley and Meziane 2002; Weiner 2004). It contributes to scrutiny of the optimal partitioning theory (OPT), which states that a plant invests always into improving the access to the limiting factor; e.g. if this is light or water, the plant invests in shoot or root growth respectively (Bloom et al. 1985). Recently, the necessity of estimating the below-ground carbon content enhanced the interest in root–shoot allometry (Levy et al. 2004), as shoot size might be used as predictor variable for root size and biomass. The root–shoot allometric exponents ($\alpha_{r,s}$ in $w_r \propto w_s^{\alpha_{r,s}}$) and root–shoot ratios $q_{r,s} = w_r/w_s$ of herbaceous and woody plants vary in a broad range between $\alpha_{r,s} = 0.2 - 1.3$ and

$q_{r,s} = 0.1 - 1.0$, respectively (Hofman and Ammer 2008; Levy et al. 2004; Müller et al. 2000; Pretzsch 2009; van Hees and Clerkx 2003). And most of the studies revealed a non-isometric root–shoot development ($\alpha_{r,s} \neq 1$), which may lead to confusion of simple size effects with plasticity (cf. Sect. 2.2). Allometric studies of herbaceous plants (Müller et al. 2000) and woody plants (Kozovits et al. 2005) attribute differences in the root–shoot ratio simply on an ontogenetic allometric size effect. According to these studies, α in $w_r \propto w_s^{\alpha_{r,s}}$ amounts to $\alpha < 1$ and values between 0.3 and 1.0; in other words, root biomass grows slower than shoot and the root–shoot ratio decreases just size dependent, i.e. allometrically (APT). Other authors (Hofman and Ammer 2008; Meier and Leuschner 2008) corroborate the OPT and find plasticity beyond allometric development. Bloom et al. (1985) and Shipley and Meziane (2002) reveal the interplay of size-dependent allometric size development and site-specific plasticity of plants; obviously, plasticity beyond allometric shape evolution decreases, when site conditions get worse and under strict water limitation, root–shoot growth is merely size dependent. As shown in Fig. 2, plants allocate biomass (and thus optimise the allometric exponent and distribution key α) not only in response to changes in size (APT), but also react on external factors like site conditions (OPT).

2.2 Detection of Changes in Allometry and Allocation Pattern

2.2.1 Elimination of the Size-Effect

In general, an allometric relation between two dimensions, e.g. $\alpha_{r,s}$ between root and shoot biomass, leads to a changing biomass ratio between root and shoot during development – except if it is isometric, i.e. $\alpha = 1$ between one-dimensional size variables and 1/3 exponent scaling between variables of quadratic and cubic dimensions. When the allocation pattern of two differently treated groups of plants (e.g. fertilised versus untreated reference) is to be compared, the mean size is often different, because of the accelerated growth in the fertilised group. This advance in size causes a purely ontogenetical difference in biomass ratios. So, comparisons between biomass ratios of the two groups run the risk to misconstrue simple size effects as changes in the allocation key caused by the fertiliser. In non-isometric allometric plant growth, each factor that changes size growth changes also the biomass ratios. In order to distinguish allocation effects from simple size effects, the size effect is often eliminated; there is only evidence of group differences when the group means still differ after elimination of the size effect.

Suppose organ y scales with an exponent of α_y to total plant weight x and organ z with α_z ($y \propto x^{\alpha_y}$, respectively $z \propto x^{\alpha_z}$). Then, the ratio between the biomass of organ y and z ($r_{yz} = y/z$) changes with size like

$$r_{yz} \propto x^{\gamma} \tag{6}$$

with $\gamma = \alpha_y - \alpha_z$.

Equation (6) reflects, that except for $\gamma = 1$, the biomass ratio changes nonlinear with size x, and that the size effect should be eliminated before interpretation of the differences as a changed allocation pattern caused by treatment. Suppose x_1 and x_2 are the mean sizes in groups 1 and 2, respectively, then – assuming the same allometric exponent and factor – $r_{yz,1} \propto {x_1}^{\gamma}$ and $r_{yz,2} \propto {x_2}^{\gamma}$ and

$$\hat{r}_{yz,1} = r_{yz,2}(x_2/x_1)^{-\gamma}. \tag{7}$$

That means, given the observed ratio in group 2 of treated plants, $r_{yz,2}$ and the size quotient between the two groups x_2/x_1, (7) enables us to derive the expected biomass ratio $\hat{r}_{yz,1}$ after any size effect is eliminated.

The following example shows the relevance of such an elimination of the size effect. Suppose the root biomass w_r scales with an exponent of 0.55 and the leaf biomass w_l with 0.75 to the total plant biomass w_t ($w_r \propto {w_t}^{0.55}$ and $w_l \propto {w_t}^{0.75}$), then for the ratio between root and leaf biomass ($r_{w_r w_l} = w_r/w_l$) applies $r_{wrwl} \propto {w_t}^{-0.20}$. If measurement would yield $r_{wrwl,1} = 1.6$ for group 1 of damaged trees and $r_{wrwl,2} = 1.5$ for reference group 2, a different allocation key between the groups seems obvious. However, if we consider that tree size in group 1 is $w_{t,1} = 400$ kg compared with $w_{t,2} = 550$ kg in group 2, we can apply (7) to eliminate the effect of the size difference and allometric change due to ontogenetic drift. The estimated ratio for group 1 would be $\hat{r}_{w_r w_{l,1}} = 1.5(550/400)^{0.2} = 1.60$. In other words, the ratio of group 1 is different from that of group 2, but behind the difference is merely the slowing down of size growth and not an altered biomass allocation key.

2.2.2 Deviation from Scaling Rules

Environmental changes can lead to deviations in the allometric development, following a constant species-specific exponent α. In plots with double-logarithmic scales, deviations from allometric behaviour are apparent by the deviations from the linear slope. For a detailed analysis of x–y-allometry, the slope α' in (3) can be calculated from the pairs of variables $y_{i=1\ldots n}$ and $x_{i=1\ldots n}$ from consecutive surveys, which are commonly available from long-term observations in forest growth and yield science. In Fig. 4, we illustrate the usefulness of slope α' for quantifying the effect of (a) competition, and (b) long-term ozone fumigation on the h–d-allometry of European beech and Norway spruce.

Figure 4a shows the h–d-allometry of European beech in the Norway spruce–European beech mixed stand FRE 813/1 near Freising in the period 1994–2005 (Pretzsch and Schütze 2005). The calculation of $\alpha'_{h,d} = \ln(h_{2005}/h_{1994})/\ln(d_{2005}/d_{1994})$ (cf. (3)) results in $\alpha'_{h,d} = 0.85$, on average, with a range of $\alpha'_{h,d} = 0.1$–3.5. This means, when diameter increases by 1%, height increases on average by 0.85%. However, thorough analysis reveals that, in case of small understory trees, a diameter increase of 1% corresponds with a height growth of

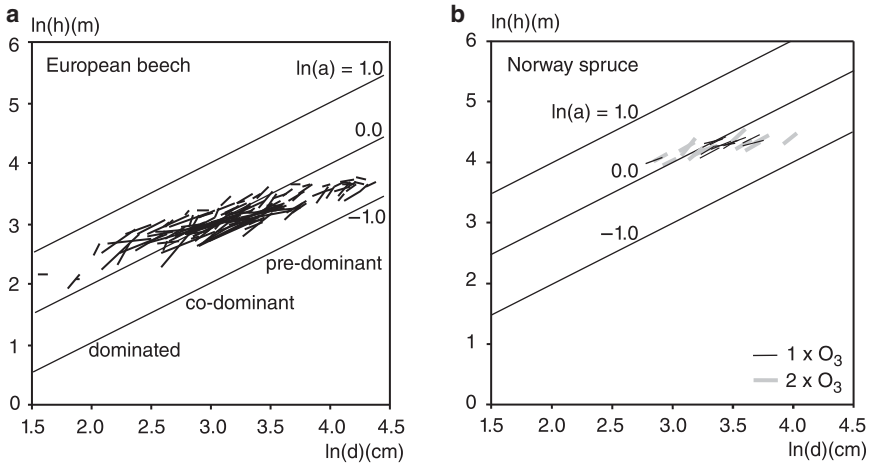

Fig. 4 Change of h–d-allometry of (**a**) European beech under competitive stress and (**b**) Norway spruce under ozone stress, shown in a double-logarithmic coordinate system (**a**) For European beeches of different social classes ($n = 107$), the h–d-allometry is shown for the period 1994–2005. (**b**) For Norway spruce ($n = 19$) trees under ambient ozone (*thin black lines*; $1 \times O_3$) show shallower slopes than trees under double ozone fumigation (*bold gray lines*; $2 \times O_3$). The *straight lines* represent the allometry expected for geometric scaling with $\alpha = 1.0$ and intercepts ln(a) = – 1.0, 0.0 und + 1.0 and serve as a reference

up to 3.5% (steep slopes on the left of Fig. 4a), whereas, in case of dominant trees, a corresponding height growth of 0.1–0.5% was found (shallow slopes in the right part of Fig. 4a).

Long-term ozone fumigation in a part of the same stand changed the *h–d*-allometry in the period 2000–2007 for Norway spruce. The canopy space of this part of the experimental plot is accessible by a scaffolding and a canopy crane, which enable annual high precision tree height growth measurement used for Fig. 4b. The thin black lines in the ln–ln-grid represent *h–d*-slopes under ambient ozone ($1 \times O_3$), bold gray lines those for $2 \times O_3$. The straight lines indicate the expected allometry under geometrical scaling ($h \propto d^{\alpha},\ \alpha = 1$). The slopes in the period 2000–2007, equivalent with the allometric exponent, range between $\alpha'_{h,d} = 0.27 - 2.42$ (mean $\alpha'_{h,d} = 0.99$). The graphical impression that those trees growing under double ambient ozone have steeper slopes can be substantiated by analysis of their allometric exponent $\alpha'_{h,d}$. The comparison between trees growing under ambient ozone concentration with sample trees under double ozone concentration in the same stand, similar in tree size, tree age and crown parameters yields significant differences of $\alpha'^{1\times O_3}_{h,d} = 0.73(\pm 0.11, n = 8)$ and $\alpha'^{2\times O_3}_{h,d} = 1.18$ $(\pm 0.17, n = 11)$. Numbers in brackets represent standard error and sample size, respectively. Obviously, under ozone fumigation, Norway spruce enhances height growth over diameter growth; in other words, its stems alter towards top-heavy, full-formed and rather unstable shapes (Pretzsch et al. 2009).

2.3 Effect of Allometric Scaling on Growth and Yield Curves of Plants

Assumptions about allometric scaling have considerable consequences for the growth and yield (accumulated growth) processes. In the sequel is shown, how the surface–weight-allometry ($s \propto w^{\alpha_{s,w}}$) affects the growth and yield curves of plants. In his attempt to explain one of the most fundamental processes of organisms' growth, von Bertalanffy (1951) describes the body mass growth rate ($\mathrm{d}w/\mathrm{d}t =$ ass – resp) as the result of two terms representing assimilation (ass $= a{\cdot}s$) and respiration (resp $= b{\cdot}w$)

$$\mathrm{d}w/\mathrm{d}t = a \cdot s - b \cdot w, \tag{8}$$

with surface area (s), plant weight (w) and a and b being species-specific factors. According to Rubner (1931), who assumed geometrical scaling, surface area s (leaf area, surface area of animal lungs or intestines) can be expressed as weight raised to the power of 2/3 ($s \propto w^{2/3}$). Assimilation is proportional to $w^{2/3}$, while respiration is proportional to weight. Consequently, (8) becomes

$$\mathrm{d}w/\mathrm{d}t = a\, w^{2/3} - b\, w, \tag{9}$$

with the generalised allometric exponent $\alpha_{s,w} = 2/3$ and species-specific allometric factors a and b. The S-shaped yield function, which results by integration of growth function (9)

$$w = A \left(1 - \mathrm{e}^{-b\, t}\right)^3 \tag{10}$$

appeared too unflexible, so that von Bertalanffy (1951) and later Richards (1959) generalised (9) to

$$\mathrm{d}w/\mathrm{d}t = a\, w^m - b\, w, \tag{11}$$

which yields in integrated form

$$w = A\left(1 - \mathrm{e}^{-b\, t}\right)^{\frac{1}{1-m}} \tag{12}$$

and is the most widely applied function for describing growth processes.

When this function is fitted to observed growth trajectories, e.g. by nonlinear regression, the exponent $1/1 - m$ reflects the allometric relationship behind the described growth process. Geometrical scaling, where $m = 2/3$ (cf. (11)) would yield $1/1 - m = 3$, which reflects that von Bertalanffy's original function is a special case of the Richards equation. For comparison, fractal quarter power scaling with $m = 3/4$ results in $1/1 - m = 4$. If regression analysis would yield $1/1 - m = 2$, that would indicate for the underlying surface–weight allometry $s \propto w^{\alpha_{s,w}}$, a half part fractal scaling, as then $m = \alpha_{s,w} = 1 - 1/2 = 0.5$.

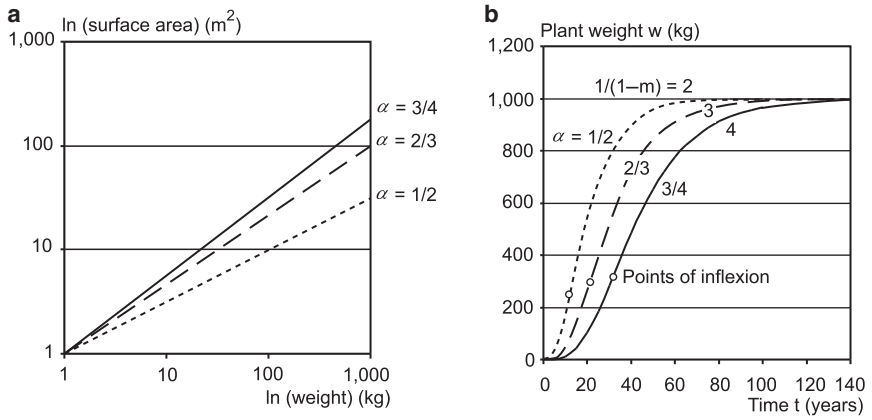

Fig. 5 Hypotheses about (**a**) the plant surface area-weight allometry $s \propto w^{\alpha_{s,w}}$ with $\alpha_{s,w}$=1/2, 2/3, and 3/4 in the double-logarithmic grid and (**b**) the resulting yield functions $y = A\left(1 - e^{-b\,t}\right)^{\frac{1}{1-m}}$ with $1/1 - m = 2$, 3 and 4, respectively

Figure 5 shows (a) for $\alpha_{s,w} = 1/2$, 2/3, and 3/4 different allometric relationships between plant weight and surface area and (b) the effect of the different scaling assumptions on the S-shape of the resulting yield curve. The biomass at the point of inflexion w_I lies at $w_I = A\,m^{(1/1-m)}$, which shows how the supposed allometry determines the form of the sigmoid yield curve. The term $w_I/A = m^{(1/1-m)}$ represents the biomass at the inflexion point relative to the maximum possible (asymptote) biomass A. It is 0.25 of the asymptote in the case of one half power scaling ($\alpha = 1/2$), and 0.296 and 0.316 in the case of geometrical and fractal scaling with $\alpha = 2/3$ or 3/4, respectively $(1/1 - m = 2,\ 3,\ \text{and}\ 4\ \text{respectively})$. Numerous analyses of growth curves with $1/1 - m$-values between 1 and 4 underline a considerable individual, species-specific and site dependent variation (e.g. Kahn 1994; Murray and von Gadow 1993) in basic allometric relationships. Suppose the three curves represent neighbouring individuals or species in a stand, the differences of their allometry and yield curves would have considerable consequences for their competition.

3 Allometry on Stand and Community Level: Linkage of Production and Growing Space Requirements

As plants grow in size, their demands on resources and growing space increase. If resources are no longer sufficient for all individuals, self-thinning commences, and the number of plants N per unit area decreases (Fig. 6). Although the principle of allometry was derived for individual plants, its application to stands or plant communities in which self-thinning occurs, is of high value in plant ecology and forestry (Enquist and Niklas 2001; Pretzsch 2002, 2006; Weller 1987, 1990; Zeide 1987).

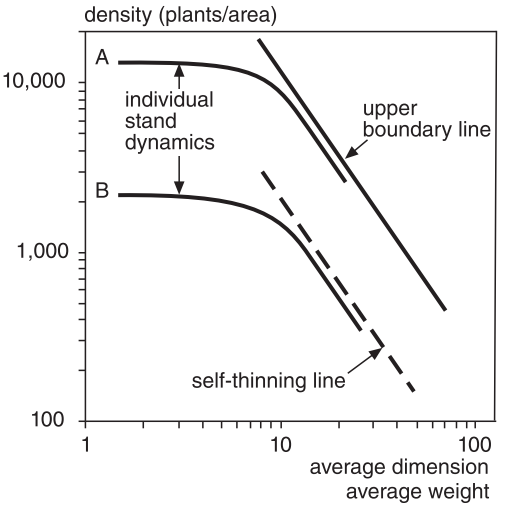

Fig. 6 The common principle of Reineke's rule (1933) and the −3/2 power law by Yoda et al. (1963) for evenaged plant populations. The relationship between average plant size or average weight and plant number forms a straight line in a double-logarithmic scale. The *upper and lower straight lines* represents the self-thinning line of the plant populations A and B and delineate the decrease of plant number under optimal respectively poor site conditions. Before crown closure the population density remains rather constant until the limited resources enhance competition and lead to self-thinning

3.1 Linkage of Production and Growing Space Requirements

Suppose the size of a certain organ, e.g. the crown projection area (cpa), shows an allometric relationship with the size of the total tree, e.g. the biomass weight w

$$w \propto \mathrm{cpa}^{\alpha}. \tag{13}$$

Let us suppose furthermore, that the crown projection area cpa represents the growing space (s) and resource requirements (r) of a tree $(\mathrm{cpa} \propto s \propto r)$. Then, (13) equals $w \propto s^{\alpha}$ or $w \propto r^{\alpha}$. As an individual's growing space or resource demand is hard to assess, the relationship was mostly postulated and analysed on stand respectively mean tree level

$$\bar{w} \propto \bar{s}^{\beta}. \tag{14}$$

with mean plant weight $\bar{w}$ and mean plant growing area $\bar{s}$. The mean growing area $\bar{s}$ ($\bar{s} = A/N$, with $A =$ unit area, e.g. hectare, $N =$ tree number) is used as surrogate variable for the mean lateral crown extension and resource demand of a plant. Thereby, (14) represents a linkage between biomass production of the mean tree and the required growing area or resources, respectively. In other words, (14) couples production ecology with population ecology (Zeide 1987).

As the average growing area $\bar{s}$ is the inverse of number of plants N ($\bar{s} \propto 1/N$), (14) can be written as $\bar{w} \propto N^{-\beta}$. Latter forms the basis of the self-thinning rule, which is shown in Fig. 6 in schematic representation on the double logarithmic scale $(\ln(\bar{w}) \propto -\beta \ln(N))$. The upper self-thinning, or limiting boundary line

(solid line), marks the maximum possible density for a species at a given average plant size, or weight in even-aged pure stands under optimum site conditions. The lower self-thinning line (dashed line) marks the characteristic boundary relationship for any stand under suboptimum growing conditions. Given two stands A and B growing under optimum and suboptimum conditions, respectively, the size–density relationships of each stand initially approximate their stand-specific self-thinning lines, and, subsequently, follow this line. The lines may have different absolute levels, but possess similar slopes (Pretzsch 2002).

3.2 Reineke's Self-Thinning Line

For the relationship between tree number N and quadratic mean diameter d_q in fully stocked, even-aged forest stands, Reineke (1933) defined the "stand density rule"

$$N = b\, {d_q}^{-1.605}. \tag{15}$$

Reineke's rule can be represented on the ln–ln scale as a straight line

$$\ln N = b' - 1.605 \ln d_q \tag{16}$$

with the intercept $b' = \ln b$ and the slope $\beta_R = -1.605$ (note that we substitute α as the allometric exponent on individual plant level by β for the stand level; the subscript R stands for Reineke). Reineke obtained this scaling rule by plotting d_q and N for untreated forest inventory plots in the USA on a double-logarithmic grid. He found very similar allometric exponents for various tree species, stand structures, and sites, and hence, concluded that the rule had a general validity of $\beta_R \cong -1.605$ for forest stands. Reineke's rule has gained considerable importance for the quantification and control of stand density, and for modelling stand development in pure (Ducey and Larson 1999; Long 1985; Newton 1997; Pretzsch 2009; Puettmann et al. 1993; Sterba 1975, 1981, 1987), and mixed stands (Puettmann et al. 1992; Sterba and Monserud 1993).

Reineke (1933) used the allometric exponent $\beta_R = -1.605$ to develop his stand density index $\mathrm{SDI} = N\,(25.4/d_q)^{-1.605}$. The SDI describes stand density in relation to the quadratic mean diameter d_q and the number of trees per hectare N by calculating the expected number of stems per hectare for a 10-inches mean diameter (= 25.4 cm; 1 inch = 2.54 cm). In Europe, an index diameter of 25 cm is used, so that

$$\mathrm{SDI} = N(25/d_q)^{-1.605}. \tag{17}$$

Stand density indices at maximum stocking densities vary according to the spatial requirements of tree species and site characteristics (Pretzsch 2009, pp. 271–273). Whereas, one can expect 900–1,100 trees per hectare with a quadratic mean

diameter of $d_q = 25$ cm in a Norway spruce stand with maximum stocking density and optimal growing conditions, and similarly high values for Silver fir and Douglas fir, the stand density indices for Sessile oak and European larch are only about the half, with 500–600 trees per hectare. SDI values of 600–750 trees ha^{-1} in stands with a quadratic mean diameter of $d_q = 25$ cm are found for Scots pine and European beech.

According to Zeide (2004, p. 7), Reineke's density assessment with the SDI "... may be the most significant American contribution to forest science...". But, Zeide like von Gadow (1986), von Gadow and Franz (1989), and Pretzsch and Biber (2005) questions the general validity of exponent $\beta_R \cong -1.605$. Last-named authors re-evaluate Reineke's rule based on 28 fully stocked pure stands of European beech, Norway spruce, Scots pine, and Sessile oak in Germany, which have been inventoried since 1870. Figure 7 shows the ln(N)− ln(d_q)-relationships for European beech, Norway spruce, Scots pine and Sessile oak. OLS regression of the model $\ln(N) = b' + \beta_R \ln(d_q)$ results in values of $\beta_R = -1.789$ for European beech, -1.664 for Norway spruce, -1.593 for Scots pine and -1.424 for Sessile oak. The allometric exponent for European beech differs significantly from the other species. There is also a significant difference between the β_R-values for Norway spruce and Sessile oak. With the exception of Scots pine, the allometric exponents deviate significantly (European beech) and almost significantly (Norway spruce, Sessile oak) on a $p = 0.05$ level from the exponent $\beta_R = -1.605$, postulated by Reineke (1933). If this species-specific allometry is ignored, serious errors in the estimate and control of density may be the consequence when using the SDI (cf. Sect. 5). Physiologically, the species-specific allometric exponent β_R demonstrates how strong a species enforces self-thinning for a given increase in diameter, or, in the words of Zeide (1985), the species' self-tolerance. According to the results from above, European beech exhibits the highest self-thinning, or lowest self-tolerance, and Sessile oak, the lowest self-thinning, or highest self-tolerance as defined by Zeide (1985).

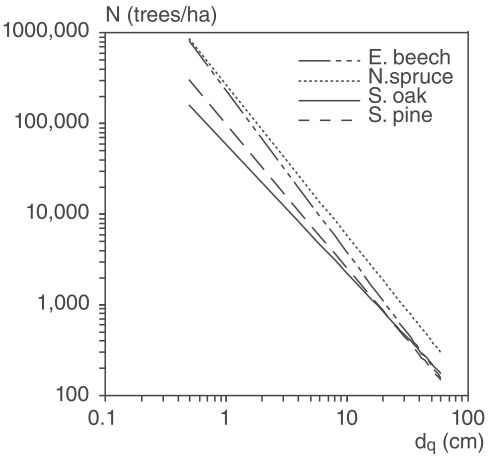

Fig. 7 Species specific ln(N)-ln(d)-relationships for untreated, fully stocked, pure European beech, Norway spruce, Sessile oak, and Scots pine stands in Bavaria/South Germany under survey since 1870 (Pretzsch and Biber 2005)

3.3 The −3/2-Power Rule by Yoda et al.

With no knowledge of the stand density rule by Reineke (1933), the Japanese scientists Kira et al. (1953) and Yoda et al. (1963) discovered the −3/2 power rule of self-thinning, initiating probably the most prominent controversial discussion of a scaling rule. It describes the relationship between the average shoot weight $\bar{w}$ and the plant number N per unit area in even-aged and fully stocked mono-specific plant populations as

$$\bar{w} \propto N^{-3/2} \tag{18}$$

with the species invariant scaling exponent $\beta_Y = -3/2$. Yoda et al. (1963) assume that plants are simple Euclidian objects, and all plant parts are related to each other isometrically. Effectively, Yoda's allometric coefficient −3/2 is based on the cubic relation between plant diameter $\bar{d}$ and biomass $\bar{w}$

$$\bar{w} \propto \bar{d}^3 \tag{19}$$

and the quadratic relation between $\bar{d}$ and occupied growing area $\bar{s}$

$$\bar{s} \propto \bar{d}^2. \tag{20}$$

As average growing area $\bar{s}$ is the inverse of number of plants N ($\bar{s} = 1/N$), (20) can be written as

$$N \propto \bar{d}^{-2} \tag{21}$$

or $\bar{d} \propto N^{-1/2}$. By inserting (21) in (19) and rearrangement, we get $\bar{w} \propto \left(N^{-1/2}\right)^3 \propto N^{-3/2}$ (cf. (18)). Equivalently, shoot biomass per unit area W scales over plant number N as $W \propto N^{-1/2}$, since $W = \bar{w}N$, $W \propto NN^{-3/2} \propto N^{-1/2}$. Equation (21) is similar to Reineke's (1933) formulation of the stand density rule, but predicts a Reineke exponent of $\beta_R = -2$ instead of -1.605.

Harper (1977, p. 183) attested the −3/2 power law, a validity for annual plants and forests as well. White (1981, p. 479) even saw the "empirical generality of the rule ... beyond question". And among others, Long and Smith (1984, p. 195) titled it "... a true law instead of the mere rule...". A quarter of the century after the first euphoria concerning the law, Begon et al. (1998, p. 169) revise their approving attitude towards the law and plead for detection of inter-specific characteristics in their allometry.

For $\bar{w} \propto N^{\beta_Y}$, Pretzsch (2006) revealed by OLS regression $\beta_Y = -1.403$, −1.614, −1.575 and −1.592 for European beech, Norway spruce, Scots pine and Sessile oak, using a broad dataset of real time series of un-thinned long-term experimental plots in Germany. The formulation of the Yoda rule with the exponent

β_Y on the side of the stem number results in an increase in the self-thinning (decrease in self-tolerance) in the order: European beech, Scots Pine, Sessile oak and Norway spruce (cf. also Pretzsch 2006).

Unlike herbaceous plants, many tree species develop a stem with a core of inert heartwood, which may comprise a considerable proportion of the tree's total biomass. For a better comparison between woody and herbaceous species, it may be helpful to distinguish between dead and living tissue. In Pretzsch (2005), functions for the estimation of biomass and sapwood area, and a stereometric model for distinguishing stem sapwood from dead heartwood are developed for European beech, Norway spruce, Scots pine and Sessile oak. The results showed that considerably smaller differences between the species self-thinning line occur with $\bar{w}'$, representing living and not total biomass. The allometry $\bar{w}' \propto N^{\beta'}$ provided values of $\beta' = -1.396$, -1.365, -1.447, and -1.369, respectively. With other words, the heartwood elimination yields a less biased slope β' and improves the comparability between the scaling rules for woody and herbaceous plants (Pretzsch 2005).

Comparison with the geometrical scaling exponent, $\beta = -3/2$ postulated by Yoda and fractal scaling slopes $\beta = -4/3$ expected by Enquist et al. (1998, 1999) and West et al. (1997, 1999) show that observed slopes β deviate considerably from the generalised exponents. However, values for β' show that the elimination of inert heartwood shifts allometry remarkably towards fractal scaling. This evaluation is not shown in order to argue for an overarching validity of the quarter (1/4 exponent) scaling, but to underline that heartwood's elimination may yield a less biased slope β', improves comparability between woody and herbaceous plants' scaling rules and paves the way for a more circumspect application of self-thinning slopes for density estimation, density control and growth prediction.

The difference in the exponents from Reineke and Yoda arises from the different allometry between quadratic mean diameter and mean plant weight. By rearranging the Yoda rule to $N \propto \bar{w}^{1/\beta_Y}$, and substituting it in Reineke's rule, $N \propto \overline{d_q}^{\beta_R}$, one obtains $\bar{w}^{1/\beta_Y} \propto \overline{d_q}^{\beta_R} \Leftrightarrow \bar{w} \propto \overline{d_q}^{\beta_Y \cdot \beta_R}$. The original exponents from Yoda and Reineke result in an exponent of $\bar{w} \propto \overline{d_q}^{2.4075}$ (Pretzsch 2009, p. 404) and Reineke's rule becomes just a special case of Yoda's. For the four species considered above, the relation produces exponents of 2.508, 2.686, 2.509 and 2.267 for European beech, Norway spruce, Scots pine and Sessile oak, respectively.

3.4 Self-Thinning Versus Alien Thinning

The slope β_{N,d_q} of Reineke's self-thinning line $\ln(N) - \ln(d_q)$ reveals the self-tolerance of a tree species growing in pure stands (Zeide 1985). The larger the β_{N,d_q}-value, the lower the number of dying trees $\Delta N/N$ for a given diameter

increment $\Delta d/d$ will be and the greater the self-tolerance of the species in pure stands (cf. (2) and (3)). The ranking we revealed for the mean species-specific, β_{N,d_q}-values European beech (-1.789) < Norway spruce (-1.664) < Scots pine (-1.593) < Sessile oak (-1.424) expresses that in comparison to Norway spruce and European beech, Sessile oak and Scots pine are more tolerant with trees of the same species. For instance, in European beech stands, a mean diameter increase of 1% causes a decrease in the number of stems by 1.79%. Given the same diameter increment, the decrease in the number of stems is 1.66%, 1.59% and 1.42% for Norway spruce, Scots pine and Sessile oak respectively, that means 7%, 11% and 21% lower than for European beech. This underlines the low self-tolerance of European beech and its space consuming investment strategy. The causes for this are its wider and more dynamic lateral crown extension, which were already discussed in Sect. 2.1 (cf. Fig. 3).

For mixed stands on comparable sites, mean β_{N,d_q}-values determined for European beech, Norway spruce, Scots pine and Sessile oak came to $\beta_{N,d_q} = -0.40$, -1.02, -1.06 and -2.01 respectively, which indicates a reversal of the ranking in pure stands, i.e. European beech > Norway spruce > Scots pine > Sessile oak (Pretzsch and Biber 2005). Compared with pure stands in which self-thinning (intra-specific) decreases tree number, in mixed stands where alien-thinning (inter-specific) occurs, β_{N,d_q} of European beech, Norway spruce and Scots pine increases, while that of Sessile oak decreases. Great crown expansion and space occupation abilities under intra-specific conditions (e.g. European beech) evidently guarantee great assertive power in the mixed stand. Low space occupation effectiveness in the pure stand (e.g. Sessile oak) is obviously combined with low assertive power in the mixed stand. This underlines, that allometry is species-specific and depends not only on size, but also on external factors (Fig. 2) and is crucial for the competitiveness and success of a species in pure and mixed stands.

4 Discussion

In the previous sections of this chapter, it was shown (1) that allometric research built up a valuable set of hypotheses and methods for analysing size of organisms and its consequences for their shape and functioning, (2) that empirical findings on organ, plant, population and community level give evidence, that allometric exponents vary in a rather narrow corridor, and (3) that it is not a single allometric exponent that carries the overarching validity but the variation within this corridor in dependence on species, environmental conditions, and resource supply. In the following discussion, the attention is drawn to methodological obstacles of allometric research, the limited benefit of hunting for general allometric rules or universal exponents, and finally to the profile of allometric research.

4.1 Methodological Considerations and Obstacles

From the broad range of methodological considerations when analysing allometric relationships and extracting allometric exponents, the following are probably the most relevant.

4.1.1 Real Versus Artificial Time Series as Source Data

The most appropriate way of analysing allometry is to record the size and form development of individuals over time by repeated measurement. By long-term measurement of the height–diameter development or by its reconstruction on the basis of stem analysis, e.g. analysis of a permanently dominant tree, we may get the dynamic *h*–*d*-allometry shown in Fig. 8a (solid line). Under stress similarity or elastic similarity, we may reveal for this tree $h \propto d^{\alpha_{h,d}}$ with $\alpha = 0.5$ or 0.66, respectively (Niklas 1994, p. 165).

However, often longitudinal data about the development of individuals or stands are not available. In such cases, the static height–diameter value pairs of a stand at a given age or in a given period (data points and broken line) are used for fitting the relationship $\ln(h) = a + \alpha \ln(d)$ and the resulting slope $\alpha_{h,d}$ is interpreted as

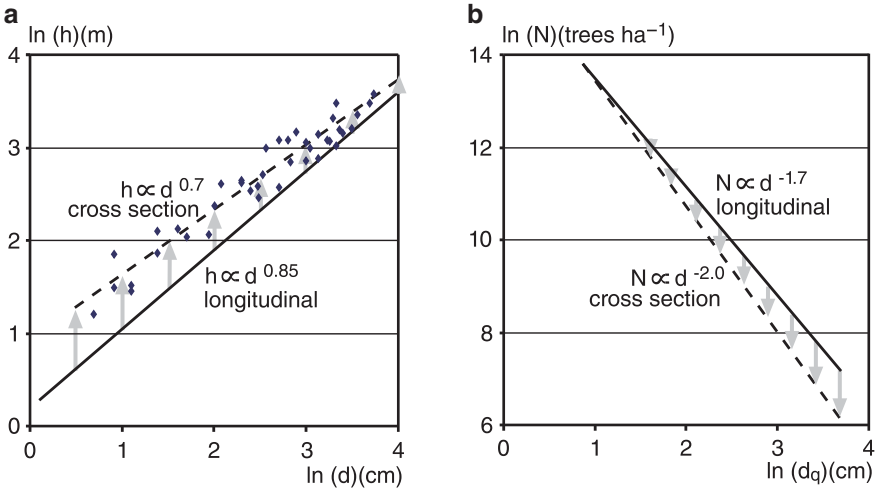

Fig. 8 Estimation of bivariate allometric relationships from longitudinal data (*continuous line* = real time series) and cross section data (*broken line* = artificial time series) in schematic representation for (**a**) the tree-height-diameter-allometry and (**b**) Reineke's self-thinning line (**a**) The slope of the cross section analysis is shallower, as suppressed trees in a stand enhance height growth (*upward arrows*) ($h \propto d^{0.85}$ *versus* $h \propto d^{0.7}$). (**b**) Slope from long-term survey and undisturbed fully stocked stands is often shallower compared with slopes from cross section inventory data, as latter includes density reduction by disturbances (*downward arrows*) ($N \propto d_q^{-1.7}$ *versus* $N \propto d_q^{-2.0}$)

allometric exponent. Such a substitution of cross section data for a real time series is often misleading as the height–diameter-relation of trees in a given period, which reflects rather the trees long-term form and shape adaptation to competitive stress than the size-dependency of their form development. In the face of competition, especially the dominated or even suppressed trees in a stand (data points on the left) increase height growth as against stem diameter growth (Pretzsch and Schütze 2005). So, the analysis on the basis of cross section data (broken line) results in a more shallow slope compared with the slope resulting from a permanently dominant tree (solid line). The two approaches are often mixed with each other, however, carefully applied they can serve to separate size dependent from competition dependent form development (Weiner 2004).

While on tree level, stem analysis or increment cores can be used to reconstruct size and shape development and reveal allometric trajectories, on stand level records of undisturbed and well-documented long-term development like those underlying Fig. 7 are very rare. So, for analysis of stand level allometry, mostly spatially adjacent stands of different ages but equal site conditions (artificial time series) substitute real time series (Enquist and Niklas 2001, Reineke 1933). However, in view of the longevity of forest stands, effects of environmental changes, disturbances like wind-throw, ice-breakage or insect calamities, which are not always documented may often be hidden in the given stand structure, so that it reflects rather the result of disturbances and adaptation than the size dependent allometric trajectory expected under undisturbed conditions.

4.1.2 Regression Techniques

When scaling exponents and scaling coefficients are computed, either Model I (ordinary least square regression, OLS) or Model II (reduced major axis regression, RMA) are applied. Zeide (1987) and Niklas (1994) argue that RMA regression (Model 2) represents the "true relationship" between the variables, as RMA slopes of x on y are exactly the inverse of those of y on x. Neither approach necessarily will produce exactly the same results, unless the $\ln(y) - \ln(x)$-pairs form a perfectly straight line. Sackville Hamilton et al. (1995) showed how slope estimates of OLS-regression, PCA and RMA-regression algorithm converge with increasing r^2 (coefficient of determination). From the examples presented in this chapter, regression yielded always $r^2 > 0.9$, and the differences between OLS and RMA slopes had no decisive effect on the final results. To take these methodological differences into account, especially when $r^2 < 0.9$, various methods should be applied to the dataset in question (Matthew et al. 1995, Sackville Hamilton et al. 1995).

4.1.3 Using Log-Transformation

The statistical analysis and extraction of allometric factors ($a, b, \ldots$) and allometric exponents ($\alpha, \beta, \cdots$) is mostly based on log- or ln-transformation of the observed

data and subsequent OLS-regression, PCA or RMA-regression. Arguments for this approach are that the effect of outliers on the result is reduced and that after transformation, the data meets the statistical assumptions of normal distribution and homoscedasticity. But logarithmic transformation involves the risk that relationships are erroneously assessed as linear as there is a linear bias of human perception, which is hard to overcome (Smith 1980). One should always keep in mind, that hardly visible differences of log-values of 4.5 and 5.0 mean a difference on the linear scale between about 32,000 and 100,000.

Analysing the self-thinning rule Zeide ((1987); Figs. 1–4) found that the $\ln(N)-\ln(d_g)$-relationship is often concave seen from below, although that is hardly visible in the double-logarithmic grid. However, application of model $\ln(N) = a_1 + a_2 \ln(d_q) + a_3 \ln^2(d_q)$ and analysis, whether a_3-values are negative or positive may reveal a concave curve, as seen from below, or a convex curve (Pretzsch 2006).

4.1.4 Transition from Plant Level Allometry to Stand or Community Level

Most studies equate without demur the allometric exponent $\alpha_{y,x}$ between the individual plant variables y and x with the respective exponent $\beta_{\bar{y},\bar{x}}$ between the same variables on mean tree or stand level. However, when individual plants follow $\alpha_{y,x}$-scaling, that does not necessarily imply, that the mean tree or stand characteristics behaves similar. If the relationship between y and x is nonlinear ($\alpha_{y,x} \neq 1.0$) and not all x-values are equal, then holds $\sum x_i^{\alpha}/n \neq (\sum x_i/n)^{\alpha}$. In other words, even if we find a constant allometric exponent $\alpha_{y,x}$ on tree level, we find a different one on mean tree level as the mean for (individual size)$^{\alpha}$ does not equal (stand mean size)$^{\alpha}$. For the relationship between leaf weight and tree diameter, Pretzsch and Mette (2008) showed that the differences between allometry on individual and mean tree level depends on the shape and development of the size frequency distribution of the stand. Blake et al. (1991) and Ford (1975) analysed the same phenomenon with respect to other plant and stand size variables.

A flawless transfer of allometry exponent α from individual level to stand level requires either $\alpha = 1$ or a steady shape of the frequency distribution of size x during the stand development. When $\alpha \neq 1$, we have to reckon with differences between plant and stand allometric exponents; the more heterogeneous and variable the frequency distribution of x, the greater is the difference between both levels of observation. Only if these differences are always of the same relative magnitude, this alters only the allometric factors (a, b, ...) and not the allometric exponents $(\alpha, \beta, ...)$. However, normally the shape of the size distribution changes over time, due to growth, loss of individuals etc., thereby causing a difference between α and β.

Since the beginning, forest science is aware of the fact that the development of arithmetic mean diameter or height of a stand has two components, the size growth of the individual trees and the increase of the mean by prevailing loss of thin trees and coupled calculative upward shift of the mean. So, the development of the mean

is an artificial course for the description of stand growth, and does not represent individual growth. In order to give mean size development a higher stability, forest science uses, e.g. the quadratic mean diameter $d_q = \sqrt[2]{({d_1}^2 + {d_2}^2 + \cdots + {d_n}^2)/n}$ instead of the arithmetic mean $\bar{d} = (d_1 + d_2 + \cdots + d_n)/n$. Especially in the case of Reineke's self-thinning law with allometric exponents close to -2, the quadratic mean stabilises the transition from tree to stand level. When y scales with a power of 2.0 to x ($y \propto x^2$), then the mean for (individual size)^2 equals (stand mean size)^2 as in this special case, when scaling exponent and weighting approach are similar, applies $\sum {x_i}^{\alpha}/n = (\sum x_i/n)^{\alpha}$ as $y_q = \sqrt[2]{({x_1}^2 + {x_2}^2 + \cdots + {x_n}^2)/n}$ and ${y_q}^2 = ({x_1}^2 + {x_2}^2 + \cdots + {x_n}^2)/n$.

4.1.5 Refined Elimination of the Size-Effect

Often, generalised theoretical or observed empirical allometric trajectories are applied for elimination of the mere size effect from a plant or stand development in order to differentiate site-specific allocation effects from ontogenetic size effects. For this purpose, a certain size measure, mostly plant weight, has to be applied as the independent variable (cf. Sect. 2.2.1). Due to practical applicability in forest research, tree diameter or stem volume is often used instead of whole tree weight. In order to scrutinize decide whether a formerly suppressed Norway spruce tree after release follows the same allocation pattern as a neighbouring open-grown spruce tree of the same size, we can use diameter or stem volume as a reference measure. However, even if the trees possess the same diameter or stem volume, they may differ considerably in other size or shape attributes (x_1, x_2, ... x_n) like crown length, crown width, root–shoot-ratio or sapwood-core wood-ratio, which also determine the future growth of the open-grown and understory tree in a very different way. Such multivariate size differences cannot be quantified by simple univariate regressions, but require multiple approaches like $\ln(y) = a + b\ln(x_1) + c\ln(x_2) \cdots + n\ln(x_n)$. At this point, we leave the reductionistic and simplistic realm of allometry and face the complexity of the real world.

4.2 Allometry as General Allocation Principle?

In the beginning, allometry and the respective methods were perceived as an approach for taking account of differences in absolute size and thereby induced changes in organ proportions, interpreting inherited, size induced form and shape developments as adaptive reactions to specific environmental conditions (Huxley 1932), and deriving growth and yield processes in dependence on first-order processes like surface-dependent assimilation and volume-dependent respiration (von Bertalanffy 1951).

Subsequently, allometric research was driven by the striving for general rules, laws or biological constants. The search stretched over organ, individual and population level aiming to reveal overarching principles, which are so rare in biology and ecology compared with physics, mathematics or chemistry. Because of their ecophysiological significance, allometric exponents have been discussed strongly ever since (Pretzsch 2000).

The most prominent Euclidean 1/3 exponents and fractal 1/4 exponents were introduced in Sects. 1.4, 3.2, and 3.3. Evidence against a universally validity of Reineke's, Yoda's or Enquist's allometric exponents was raised in this chapter and is supported by solid literature (Yoda: Harper 1977, p. 183; Long and Smith 1984, p. 195; Weller 1987, 1990; Sackville Hamilton et al. 1995, Reineke: Pretzsch and Biber 2005; von Gadow 1986; del Río et al. 2001; WBE: Whitfield 2001; Kozlowski and Konarzewski 2004; Reich et al. 2006).

The author's perception of allometry corresponds with Zeide's (1987, p. 532) conclusion after analysing Yoda's rule: "... unlike the fixed value of −3/2, the actual slopes convey valuable information about species ... that should not be cast away", or Weller's (1987, p. 37) statement that "The differences among slopes may provide a valuable measure of the ecological differences among species and plants, and a powerful stimulus for further research". As long as allometry searches for universal constants, it may still our innate propensity to reduce complexity and generalise. However, in my opinion, the mere hunting for general rules or laws hardly contributed to a better differentiated understanding of plants and stands, but it rather maneuvred allometric research somehow into a l'art pour l'art phase and isolation from related disciplines.

4.3 Changing Profile of Allometric Research

With respect to spatial and temporal resolution, the allometric approach to plant and stand growth lies between a simple statistical description of relationships and a deeper mechanistic causal explanation. Due to its simplistic approach (striving for overarching exponents and leaving the rest as white noise), the results of allometric research are difficult to integrate in both, the system knowledge of neighbouring disciplines as well as practice. While neighbouring disciplines like plant physiology need differentiated and not general explanatory schemes, also for practical purposes universal laws may yield first-order estimates, but are surely not sufficiently precise for hard economic decisions (cf. Sect. 5). Model hypotheses for data interpretation or prognosis models for use in practice, for instance, do not employ such generalised exponents.

The numerous falsifications showed that the allometric exponents tend to lie in a narrow corridor, but can be species-specific or even provenance-specific and site-dependent. The individual species' scaling exponents and their relative position in the corridor are the keys for understanding the species' ability to cope with the site conditions and competitive situation and should not be cast away, although

generalisation across species is tempting. In this sense, falsification trials concerning the rules from Reineke, Yoda and West, Brown and Enquist are turned into a refined understanding of individual species allometry, and overarching scaling exponents would appear as a stimulating myth.

Recent approaches to link APT with OPT may change the profile into a more pluralistic approach aiming at an integrated understanding from both, the inherited evolutionary optimised size induced form as well as the shape development caused by the adaptive reactions on specific environmental conditions, which are revealed under selective pressure (Fig. 2).

5 Conclusions

5.1 Avoidance of Practical Application of Inaccurate and Imprecise General Scaling Rules

Crown diameter–stem diameter allometry on individual tree level, e.g. unmasks that European beech is more expansive and space consuming with increasing size compared with the cone-like vertically growing Norway spruce. On stand level, this is reflected by a more rapid self-thinning in pure beech stands and a more moderate decline of competing neighbours in Norway spruce stands. European beech's superior gap dynamic and shade tolerance means competitive strength facing other neighbouring species in mixed stands. However, in pure beech stands, this rigorous space occupation is directed against members of the own species.

The argument that rough general rules are required for scaling from organ to community level again is tempting. However, for example the general assumption of Enquist and Niklas (2001) shows that tree number scales as -2 power of mean tree diameter can cause considerable flaws in modelling and prognosis.

Suppose in a juvenile Sessile oak stand with a mean diameter of 1 cm and a tree number of 22,027 per hectare, self-thinning starts off with the generalised slope of $\beta_{N,d_q} = -2.0,$ then $N = 22,027\ {d_q}^{-2.0}$ yields three oak trees per hectare in the mature stand with mean diameter $d_q = 80$ cm. Compared with this, the empirically found species-specific slope $\beta_{N,d_q} = -1.424$ predicts 43 trees per hectare, which is more than ten times of the theoretical stand density and more in accordance with silvicultural experience. Such differences matter when silvicultural prescriptions are based on scaling exponents.

5.1.1 Stand Density Management Diagrams as Silvicultural Prescriptions

In view of the individual species' slopes, stand density estimation algorithms, founded on generalised allometric relations, appear unsuitable. Stand density management diagrams (SDMD), which are applied for many species as tool for

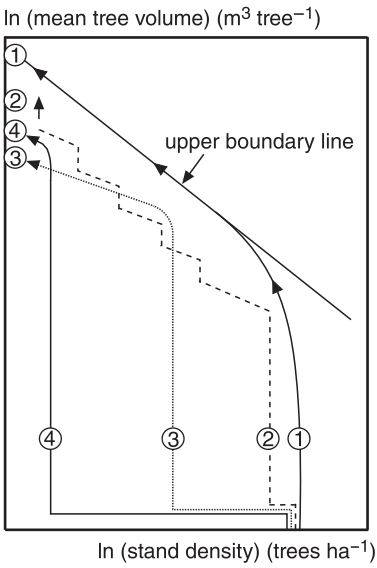

Fig. 9 Stand density management diagram (SDMD) for boreal conifer stands with four trajectories representing various thinning concepts (adopted by Weetman 2005, p. 7). The upper boundary line (*black continuous line*) represents the maximum stand density. Trajectories 1–4 all begin with dense natural regeneration, and subsequently quantify different silvicultural prescriptions. Note the logarithmic scale of the axes

regulating stand density, use the self-thinning line as upper boundary and are the most prominent silvicultural application of the self-thinning rule (Oliver and Larson 1996, pp. 352–353).

Figure 9 shows a stand density management diagram (SDMD) for boreal conifers (adapted from Weetman 2005, p. 7) with four different thinning regimes quantified by the stem number–mean volume trajectories (shown in the ln–ln-scale). The upper boundary line (solid black line) represents the maximum stand density under self-thinning with a species-specific allometric factor and exponent. The area below the line gives the scope for possible tree number–mean volume relationships. All the four trajectories 1–4 commence with dense natural regeneration, but subsequently represent different stand densities. Trajectory 1 describes un-thinned stand development yielding high total volume at low cost, but with a low mean tree volume. Trajectory 2 describes moderately dense stands established by frequent light commercial thinning to maximise volume production. In trajectory 3, a moderate pre-commercial thinning at early stand age reduces the high initial density to about half of the tree number. Trajectory 4 applies a heavy pre-commercial thinning, reducing the tree number to nearly the final level. In this context, pre-commercial is used as attribute for thinnings, which are executed in a stand development phase when stem dimensions of the removed trees are still so small that revenue does not yet cover the expense.

Bégin et al. (2001) list for a considerable number of tree species available SDMDs as guides for stand management. When those SDMDs ignore species-specific allometry but apply generalised scaling exponents, flawed density control and counter-productive thinning can result.

5.1.2 Stand Density Index by Reineke

The strength of SDI as a measure of density (cf. (17)) is that it takes mean diameter and number of tress into account (Zeide 2004). The mean diameter serves as an expression of the allometric size development of the mean tree and stand. The pure size-dependent change of tree number is eliminated by the SDI, and makes it a suitable measure for density evaluation and for comparison of stands in different stand development phases. The underlying approach for elimination of size-effects on density (cf. (17)) is analogous to the approach introduced for individual tree level in Sect. 2.2.1 (cf. (7)).

The disadvantage of the SDI is that for elimination of size-effects, it generally assumes the coefficient $\beta = -1.605$ to be valid. Wherever this allometric coefficient fails to apply, there will be severe errors in stand density estimates. This chapter shows that in untreated fully-stocked pure stands of European beech, the β-value is lower than the generalised Reineke value $\beta = -1.605$. Although the deviations from -1.605 are only close-to-significant for Sessile oak and Norway spruce, our results concerning differences among species suggest that at least one of these species' slopes differs from Reineke's generalised value, too.

If species-specific allometry is ignored, serious errors in the estimate and control of density when using the stand density index $SDI = N(25/d_q)^{-1.605}$ may be the consequence (Pretzsch and Biber 2005). The use of Reineke's rule and SDI with $\beta = -1.605$ for determination of maximum density in planning (Sterba 1975, 1981, 1987), control of stand density in the course of thinning operations or modelling of stand development (Pretzsch et al. 2002, 2008) is therefore called into question. For the SDI in Norway spruce stands, Sterba (1981), using the Bavarian yield tables for Norway spruce by Assmann and Franz (1965) and assuming $\beta = -1.605$, calculated mean values of SDI = 970, 1,081, 1,203 and 1,336 for sites with 28, 32, 36 and 40 m top height (tree height associated with the quadratic mean diameter of the 100 tallest trees in a stand) at age 100 years. These values are quoted here to illustrate the extent to which values may be biased when erroneous β-values are being used. Assuming we determine, according to Reineke, the SDI of a stand with mean diameter 10 cm to be 1,300, assuming further that this stand actually follows a straight line with $\beta = -1.805$, then the determined SDI of 1,300 would have to be reduced by $k = 0.83$. The correction of the bias would therefore signify a shift of values from the upper end (SDI = 1,300) to the lower end (SDI = 1,079) in the range of SDI values observed in Central European Norway spruce stands (Pretzsch and Biber 2005).

5.2 Research Perspectives

In comparison with ecophysiological and biochemical processes, which are far from being thoroughly understood, size and structure of plants are much easier to measure. Since there is a close feedback between structure and process, organisms'

size and structure can become the key for revelation and prognosis of stand dynamics. Due to the uncomplicated possibility of their spatial explicit measurement (e.g. compared with grassland and agricultural crops), organs of forest trees, whole trees, stands and communities are best suitable for revealing and tracing allometry over various levels of system organisation.

As provisional approaches for urgent upscaling climate change C-storage estimations for instance, even imprecise and inaccurate allometric relationships may be helpful. However, rest and faith in such general scaling rules would pass up the opportunity of a better understanding of ecosystem structure and functioning. Rather than continuing to search for "*the ultimate law*", further research should exploit the potential, which lies in a differentiated allometry between species, site conditions, competition, disturbances etc. in order to systemise it, integrate it in system knowledge and apply it for mechanistic understanding of plant growth.

One focus might be on a systematic revelation of plant and stand allometry with regard to different species, provenances, clones, and under varying site conditions, competitive status in pure and mixed stands and under disturbances. The concept, methods and at least a part of the data base for such an inventory are available. Methodological shortcomings of previous analysis like neglection of the dead inner core of trees, equation of individual and stand allometry, univariate description and imperfect elimination of size-effects can be considered. Maybe a plant's or stand's allometry reveals rather its tricks and traits of optimising fitness in relation to neighbours or other stands than a random deviation from a general rule. A systematic analysis, ordering and causal explanation of different allometry exponents is missing so far. A closer analysis of the species-dependency of allometric exponents and their determination by internal and external factors might provide an important link between plant genetics and physiology on the one and plant and population biology and morphology on the other hand. Allometric research should strive for a closer link to plant physiology and modelling to integrate the existing knowledge in order to find mechanistic links, causal explanations. Closer connection with stand ecology and forest dynamics would help to separate between internal and external factors and avoid to misinterpret the effect of disturbances as allometric patterns.

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Quaking Aspen's Current and Future Status in Western North America: The Role of Succession, Climate, Biotic Agents and Its Clonal Nature

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Contents

Abstract Quaking aspen (*Populus tremuloides* Michx) exerts significant influence on the function and diversity of boreal and subalpine forests of western North America. Aspen's expansive range highlights its success in adapting to a variety of environmental conditions. Recent patterns of dieback and habitat loss suggest that shifts in environmental conditions appear to be placing constraints on aspen vigor in

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some, but not all portions of its western range. The objectives of this chapter are to outline recent trends in aspen's status in western North America and to establish a physiological framework for understanding current and future trends in aspen ecology in the context of succession dynamics, shifts in climate conditions and biotic factors, and aspen's clonal nature. The literature suggests that aspen decline is occurring in some areas but that trends are highly variable depending on: site characteristics, fire and succession, extreme climatic events, biotic agents, and human influence.

1 Introduction

Quaking aspen (*Populus tremuloides* Michx) is a keystone tree species in North America, where it exerts a significant influence on the function of subalpine and boreal forest systems. Aspen ecosystems are characterized as having high biodiversity that supports a variety of forb, grass, and shrub species (Hollenbeck and Ripple 2007; DeByle 1985) and a wide diversity of mammals, birds, and arthropods throughout its range (Stohlgren et al. 1997; Mueggler 1985b). The high productivity and structural diversity of aspen communities, creates habitat and forage that is critical to both wildlife and livestock (DeByle 1985). Emerging evidence suggest that aspen dominated watersheds may produce significantly greater water yields than those dominated by conifers, which has important implications on water supply in the semi-arid regions of western North America (LaMalfa and Ryel 2008; Gifford et al. 1984). Healthy functioning aspen forest can ameliorate atmospheric perturbations by serving as sinks for atmospheric CO_2 (Barr et al. 2007; Griffis et al. 2004) and by buffering regional temperature and precipitation patterns (Balland et al. 2006; Hogg et al. 2000a).

Quaking aspen has a clonal growth habit where suckers arise vegetatively from an existing parent root system. Clonal regeneration in aspen is particularly common in its western range and clones can be as large as 81 ha in size and have as many as 100,000 stems (Kemperman and Barnes 1976). Because of their dominant ecological role in both boreal and mountain forests in western North America, their heritable physiological, growth, and regeneration traits exert significant influences on the function and structure of these ecosystems. There is a need to better understand the factors that influence aspen's regeneration success and the ecological implications of aspen's clonal nature.

Aspen is the most widely distributed tree species in North America, extending across diverse topographical, edaphic, temperature, and moisture gradients (Jones 1985). Its success across such a broad range attests to its resilience and adaptability to a wide range of environmental conditions. However, recent patterns of aspen decline and dieback suggest that current management strategies and changing environmental conditions may impose constraints on aspen vigor across portions of its western range (Fig. 1). Yet, there are critical knowledge gaps regarding the extent and causes of aspen decline. In this chapter, key insights have been

Fig. 1 Locations of reported aspen dieback, defoliation and decline (*shaded black*) within the range of aspen in the western US (*gray*). Data are taken from USDA Forest Service, Forest Health Protection aerial survey from 2002 to 2008 (Anonymous 2007)

synthesized from the literature to better define the current status of aspen in its western range, to identify traits that explain its success (as defined by its dominant nature and broad geographical range), and environmental factors that are reducing aspen fitness and contributing to patterns of dieback and habitat loss. The objectives of this chapter are to outline recent trends in aspen's status in western North America and to establish a physiological framework for understanding current and future trends in aspen ecology in the context of succession dynamics, shifts in climate conditions and biotic factors, and aspen's clonal nature.

2 Aspen Decline and Dieback

Many authors have discussed aspen decline referring to several different phenomena, from succession to conifers due to fire exclusion (Mueggler 1989), the predominance of older stems in clones and stands due to browsing/grazing pressure (Kay 1997), regeneration failures (Hinds and Shepperd 1987), long and short-term climatic trends (Hogg et al. 2008), extreme weather events (Fairweather et al. 2008), and insect and disease activity (Worrall et al. 2008). Forest inventory and analysis (FIA) data, suggest decreasing aspen cover in some regions of its western range (Rogers 2002; Bartos and Campbell 1998). Aspen dieback has been observed from western Canada (Hogg et al. 2008) south through the Rocky Mountains (Bartos and Campbell 1998), including recent, large-scale, aspen mortality events in southwestern Colorado (Worrall et al. 2008). However, studies conducted in two regions of Colorado's western range found little evidence of aspen decline during the twentieth century (Kulakowski et al. 2004, 2006), suggesting that incidence of aspen decline varies regionally, as do the proximal causes of the dieback/decline. Several authors (Kulakowski et al. 2004; Manier and Laven 2002) argue that while succession to conifers is occurring, aspen has actually increased in presence, if viewed in the context of longer time scales. A major limitation in determining the extent and relative importance of aspen dieback and habitat loss is that the studies assessing these trends are relatively few in number and have occurred on spatially limited scales (Rogers 2002). Clearly, there is regional and even local variation in aspen status that varies depending on site conditions with no single trend applying to aspen forests in general (Kashian et al. 2007).

Aspen dieback/decline is currently receiving a lot of attention, but this phenomenon is not new. Declining aspen forests were noted as far back as 1925 (Baker 1925). During the 1970s, widespread deterioration of aspen was observed in the Great Lakes region, where high mean annual temperature was considered to be an important causal factor (Shields and Bockheim 1981). Stands with open canopies had increased solar insolation of the forest floor, wind exposure, and moisture loss, which may have contributed to symptoms of health decline (Fralish 1975). Pathogen activity and defoliating insects were also contributing factors (Katovich and Hanson 2001). The aging of stands was also considered important, particularly after ramets were greater than 60 years in age (Fralish 1975). Similar decline was observed in the western US around the same time (Schier 1975). One important difference is that the stands in the west are often older due to logging and fires in the upper Midwest and eastern US. Mueggler (1989) found that 75% of Intermountain aspen were in the 80–120-year-old age classes.

More recently, a phenomenon known as SAD (sudden aspen decline) has been occurring in southwestern Colorado (Worrall et al. 2008). SAD is differentiated from other types of aspen decline not only by the sudden onset, but also by lack of suckering response often linked to death of the root system. The overlapping of multiple factors further complicates the picture. The model of a decline type disease has been proposed by some authors (Frey et al 2004; Guyon 2006) as a conceptual

framework to help in explaining the overall impact of the multiple interacting factors involved in aspen decline. Episodic dieback and regeneration does appear to be part of the life history of aspen forests, but the scale of current dieback and regeneration failure appears to be unprecedented. An examination of the primary drivers of recent aspen dieback/decline in different areas across western North America can illuminate the diversity of conditions and factors associated with this phenomenon.

2.1 Yellowstone Area

A great deal of both anthropological and natural history research has been aimed at understanding the biology, historical importance, and distribution of aspen in the greater Yellowstone area. Kay (1995) has argued that man was the keystone species in Yellowstone and surrounding ecosystems in the nineteenth century, and that aboriginal burning and hunting were the driving forces behind the distribution and abundance of aspen and other vegetation types. Some have questioned Kay's methods (see Yochim 2001), nevertheless, many studies indicate that aspen recruitment (regeneration) peaked in the late nineteenth and early twentieth centuries, corresponding with historically low elk populations due to high levels of hunting pressure (Meagher and Houston 1998; Romme et al. 1995). Since 1920, when elk were protected, and after predator populations had been decimated, aspen has shown virtually no regeneration over vast reaches of the Yellowstone area.

The 1995 reintroduction of wolves into Yellowstone, has provided additional data to suggest elk's importance in aspen's regional decline. Wolves are a keystone predator in Yellowstone and their reintroduction has led to a trophic cascade via their effect on elk populations (Ripple et al. 2001). By limiting ungulate populations, increasing the frequency of elk movements, and changing elk feeding behaviors, wolves indirectly alter the level and intensity of browsing pressure on aspen suckers. Since wolves were reintroduced to Yellowstone, aspen is successfully regenerating in many areas where it was previously in decline (Halofsky and Ripple 2008; Ripple et al. 2001).

2.2 Utah and Nevada

Researchers have been concerned about aspen decline in the Intermountain West for many years (Bartos and Campbell 1998), but recently dieback and defoliation has been evident on a large enough scale to be detected by aerial survey. To determine the causes of this dieback and defoliation, we recently conducted a survey in the Utah and Nevada, as well as Southern Idaho, and western Wyoming. Full data analysis has yet to be conducted, but a few preliminary trends were evident.

The survey sampled all age classes, and it was found that younger stems were damaged by a host of agents. Grazing damage was the most common, occurring on 68% of all plots. Defoliator activity was noted in some areas on all size classes, particularly in the Dixie National Forest, and areas in the Humboldt–Toiyabe National Forest, primarily caused by three agents, large aspen tortrix (*Choristoneura conflictana*), Aspen leaf tier (*Sciaphila duplex*), and Aspen two leaf tier (*Enargia decolor*). Two insect borers, Poplar borer (*Saperda calcarata*) and Bronze poplar borer (*Agrilus liragus*), and two canker diseases, Cytospora canker (*Valsa sordida*), and sooty bark canker (*Encoelia pruinosa*) were the most common damaging agents found on trees over 10 cm in diameter. Of these four agents, all, except sooty bark canker, are commonly considered as stress related pests (Hinds 1985). The area under study has been in a drought since 2000, which may be sensitizing the aspen to the observed biotic agents. Sooty bark canker is usually considered as a disease of older stands (Hinds 1985). Collectively, this suite of damaging agents paints a picture of stressed, older aspen with high rates of mortality and damage under considerable grazing pressure (Table 1).

A few differences from the Colorado survey conducted by Worrall et al. (2008) are noteworthy. First, while aspen bark beetles (*Trypophloeus populi* and *Procryphalus mucronatus*) were detected in our survey, they were generally found only on trees that already had canker diseases, and were rarely considered to be the primary damaging agent. Second, sooty bark canker was much more common, perhaps indicating older stands. Third, and most importantly, most of the Intermountain stands showed at least some suckering, indicating that the root systems were not moribund (Table 2).

Table 1 Percent mortality and damage of large trees in plots from a survey of National Forests in Utah, Nevada, Idaho and western Wyoming showing evidence of dieback or defoliation

National forest	Mortality	Damage
Dixie	28	59.2
Uinta	30.2	23.1
Manti	20.9	56.1
Wasatch-Cache	33.3	33.3
Fishlake	23.5	29
Bridger-Teton	45.9	57.6
HT Jarbidge[a]	28.6	50.3
HT-Rubies	28.9	47.1
H-T Austin	25.5	45
Caribou	24.6	24.2
Targhee	28.5	28.6
Sawtooth	21.5	32.4

[a]Humboldt-Toiyabe NF districts

Mortality was estimated to have occurred within the last 5 years. Damages percentage was rated as moderate or severe. Light damage was not included

Table 2 Average number of trees less than 5 cm in diameter per hectare from a survey of National Forests in Utah, Nevada, Idaho and western Wyoming showing evidence of dieback or defoliation

Uinta	1,030
Manti-LaSal	13,830
Fishlake	8,768
Wasatch-Cache	10,620
H-T Ruby Mtns[a]	3,580
H-T Austin	4,475
H-T Jarbidge	8,300
Caribou	4,780
Targhee	2,800
Sawtooth	13,500
Bridger-Teton	11,800

[a]Humboldt-Toiyabe NF Districts

2.3 Idaho and Western Wyoming

Mortality and damage from insects and diseases were not as severe in Idaho as in Utah and Nevada (Table 1), and grazing pressure was neither as common nor as damaging. Despite the relatively low pressure from grazing, many of these stands still had fairly low presence of suckers (Table 2), while others had a suckering response that reflected the response expected from healthy stands that had recently experienced an overstory disturbance. However, many of the Idaho plots had considerable competition from understory grasses, forbs, and shrubs, indicating lack of fire. In fact, this lack of understory disturbance may be the biggest limiting factor controlling regeneration in the plots with limited suckering response. In the case of the Idaho stands that are not regenerating, apical dominance has only been partially broken and competing vegetation in the form of shrubs and other plants is shading the young suckers, particularly on the Targhee National Forest.

2.4 Colorado

Southwestern Colorado currently is experiencing dramatic aspen dieback/decline with over 56,000 ha of aspen decline detected in aerial survey in 2006 and 32% average mortality. Researchers there consider many stands to be experiencing SAD (Worrall et al. 2008), with high rates of overstory mortality and little or no replacement by sucker sprouts. Worrall et al. (2008) described the situation as similar to a forest decline outlined by Frey et al. (2004): "The proximate cause was a group of interchangeable, usually secondary, biotic agents. There has been little regeneration response and roots in some stands may be moribund. Relative to healthy aspen, recent mortality occurred at lower mean elevations and on flatter slopes, affected larger tree sizes, was associated with stands of low density, and

tended to occur on southwestern aspects in some areas and on eastern to southern aspects in other areas." Sooty bark canker was less common than in Utah, Nevada, and Idaho, and aspen bark beetles were more common and more damaging. Researchers hypothesize that several years of drought may be an important factor contributing to SAD (Worrall et al. 2008).

2.5 Canada

Aspen dieback and decline has been extensively studied in Canada, particularly in the Prairie Provinces. This research is summarized by Frey et al (2004), who called the situation "dieback," but described it using the terms of a forest decline (Manion 1991). They describe dieback as involving aboveground death of ramets, often rapid, but with partial survival of the root system, which has the potential to regenerate the stand. They describe a number of features of this dieback operating at a wide range of temporal and spatial scales.

Canadian researchers have installed an extensive plot system to monitor aspen, the CIPHA system (Hogg et al. 2008). Their analysis showed that net mean increment in living biomass collapsed following a 2000–2002 drought (Hogg et al. 2008), and that this collapse was primarily driven by regional drought stress, measured as mean annual CMI (climatic moisture index). Mortality and dieback were most closely tied to minimum annual CMI, which provided a measure of short-term drought severity. The proximal causes of this mortality were primarily due to drought stress, and the activity of forest pests, most notably forest tent caterpillar (*Malacosoma disstria* Hubner) and poplar borer. The stands involved in the CIPHA system are primarily younger than most Intermountain aspen (Frey et al. 2004; Mueggler 1989). Ungulate grazing is largely not a driving force in these studies (E.H. Hogg, personal communication), but can be in other areas in Canada (White and Feller 2001; MacIssac et al. 2006).

Forest tent caterpillar activity has been reported in Canada and elsewhere for many years (Blais et al. 1955), and has been associated with defoliation and dieback, including some long-term impacts, such as growth loss and dieback (Churchill et al. 1964). Hogg et al (2002) reported that defoliation by forest tent caterpillar and drought in the 1960s and 1980s led to reduced growth and predisposed some stands to secondary damage by wood-boring insects and fungal pathogens.

3 Aspen Succession

3.1 Aspen's Seral and Stable States

Aspen typically regenerates through root suckering following disturbance (Paragi and Haggstrom 2007; Fraser et al. 2004), and can dominate a site on decade to century time scales. In the absence of disturbance, aspen is often seral to conifers or

hardwoods (Bergen and Dronova 2007; Rogers 2002). However, stable aspen stands that self-regenerate in the absence of disturbance are not uncommon, and in some cases, aspen shows evidence of multiple age cohorts that are not linked to disturbance (Kurzel et al. 2007). Large proportions (>30%) of aspen stands surveyed in Colorado were found to persist in a stable state without significant establishment of conifers (Kurzel et al. 2007; Kaye et al. 2003). One of the important questions in aspen ecology is what are the factors that define aspen's successional status? For conifers to establish in aspen stands, a seed source is required, but whether seed arrival limits conifer establishment is poorly investigated. If conifer seeds do arrive, what are the constraints for successful germination or establishment? Aspen stands support a wide diversity of understory plant species, often at densities that could have competitive impacts on conifer seedling establishment and survival (Mueggler 1985a).

3.2 The Role of Fire and Climate

Fire return intervals have a major impact on aspen–conifer succession dynamics. Recent studies on fire history in subalpine and boreal forests, suggest that both climate conditions (Beaty and Taylor 2008; Buechling and Baker 2004) and fire suppression by humans (Van Wagner et al. 2006; Gallant et al. 2003) has lengthened fire return intervals during the last century. Grazing also plays a role in lengthening fire return intervals by reducing fine fuels (Jones and DeByle 1985). Studies suggest that increases in the length of fire intervals have reduced aspen cover, while increasing conifer dominance (Smith and Smith 2005; Gallant et al. 2003; Bergeron 2000). Studies in Colorado's western range showed little evidence of aspen succession to conifer at low to mid elevations, but some succession was observed at higher elevations (Kulakowski et al. 2006). These studies taken together suggest that while aspen–conifer succession patterns are spatially variable, trends of aspen succession to conifer are occurring as disturbance return intervals increase.

Future climate scenarios are likely to have a major impact on fire's influence on aspen regeneration. Intense fire years in aspen–conifer forests have coincided with severe droughts (Margolis et al. 2007; Fule et al. 2003). Climate projections in aspen's western range are for warmer and drier conditions and increasing frequency in severe droughts in the coming decades (IPCC 2007; Seager et al. 2007). Drier conditions are likely to result in shorter fire return intervals, which will tend to trigger aspen regeneration. A drier climate and higher fuel loads (a product of fire suppression) favor more expansive and greater intensity fires, which stimulate aspen suckering more than low severity burns (Keyser et al. 2005; Fraser et al. 2004). Bark beetle outbreaks resulting in a greater proportion of dead conifers may also hasten fire return intervals and increase burn intensity (Bigler et al. 2005). However, gains in sucker stimulation that may occur from more frequent and greater intensity fires may be offset by reductions in sucker initiation and growth under conditions of drought (Frey et al. 2003).

3.3 *Conifer Establishment and Impacts on Aspen Fitness*

Aspen does not burn well (Jones and DeByle 1985), and is dependent on the presence of fire prone conifers or shrubs to produce a mixed stand that is susceptible to burning (Cumming 2001). Many conifer species establish better under aspen stands than in full sunlight or in deeper shade found beneath conifer canopies (Gradowski et al. 2008; Shepperd and Jones 1985). It has been found that subalpine fir (*Abies lasiocarpa*) established more readily and achieved taller height classes when the overstory canopy had aspen as a dominant component (Fig. 2). Aspen facilitation of conifer establishment promotes mixed stands that are more susceptible to fire. However, as fire return intervals lengthen with fire suppression, understory regeneration success becomes increasingly more important in determining aspen's persistence in the landscape. The abundance and frequency of aspen regeneration is linked with genet survival under longer fire return intervals (Namroud et al. 2006). Aspen can continue to self-regenerate in the absence of disturbance, however aspen regeneration decreases markedly as conifer succession progresses (Kurzel et al. 2007; Smith and Smith 2005).

Conifer encroachment into aspen stands has significant effects on mature aspen tree growth and regeneration success through changes in light conditions. Leaf area index (a measure of canopy light interception) is greater in mixed and pure conifer stands than in pure aspen stands (Fig. 3). Aspen is shade intolerant relative to conifers (Kobe and Coates 1997), which is partially related to its shoot characteristics, which are optimal for growth under full sun conditions, but constrain growth potential in shaded environments (Messier et al. 1999). However, there is a significant amount of light heterogeneity under aspen–conifer canopies (as indicated by the large error bars in Fig. 3), which is largely the result of canopy gaps. Hill et al.

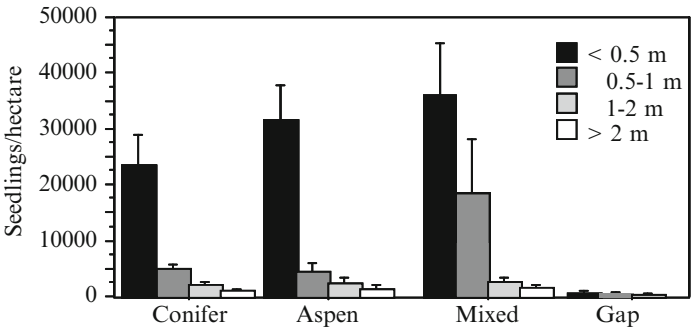

Fig. 2 Influence of overstory canopy composition on subalpine fir establishment and height classes (*different colors*). Greater establishment and taller height classes are achieved when aspen is a significant component of the overstory. Data was collected from five field sites in mature stands on the Fishlake National Forest in central Utah. 50 m transects were run in four overstory zones (Conifer $\geq$ 80%, Aspen $\geq$ 80%, Mixed 50/50 aspen/conifer, and gaps lacking an overstory) to characterize the overstory influence on subalpine fir regeneration success. Unpublished data

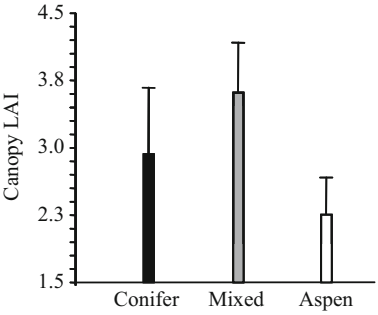

Fig. 3 Mean leaf area index values (a measure of canopy light interception) in mature pure aspen (>90%), conifer (>90%) or mixed aspen–conifer stands (50% of each). Note the substantial variation in means as denoted by the large standard error bars. This was largely a function of large numbers of canopy gaps. Data was collected from seven field sites on the Fishlake National Forest in central Utah. 50 m transects were run in the three overstory zones and leaf area index values were determine every 5 m along the transect. Unpublished data

(2005) found that in the absence of fire, canopy gaps are created and expand in boreal aspen stands. Based on its shade intolerance, aspen regeneration would tend to benefit from canopy openings where light levels are high. Messier et al. (1999) concluded that canopy gaps would have to be relatively large to sustain aspen under a conifer cover type. These studies highlight the potentially important but poorly understood role of gap dynamics in patterns of aspen–conifer succession. While, substantial research has focused on aspen regeneration in response to disturbance cycles, future research needs to focus on regeneration patterns under gap dynamics particularly in the later stages of aspen to conifer succession.

In addition to changing light conditions, conifer encroachment into aspen stands can alter soil chemistry (Cryer and Murray 1992). A broad sampling of FIA plots, show that aspen soils tend to have lower bulk densities, and are higher in soil organic matter and nutrients than soils under conifer stands (Personal communication, Michael Amacher). We conducted a study to examine how differences in soil chemistry and light environment affect aspen and subalpine fir growth and function. The aspen and subalpine fir were grown on soils collected under pure aspen or pure conifer stands and in low or high light conditions. We found that aspen had substantially greater reductions (> 50%) in photosynthesis and growth rates than subalpine fir when grown under low light or on conifer soils (unpublished data). These results underscore the important role of light and soil nutrient limitation on aspen regeneration vigor as conifer succession advances.

In summary, longer fire return intervals promote conifer establishment, which over time alters soil and light conditions and promotes competition between aspen and conifers. The sum of these changes are decreases in aspen overstory growth rates (Shepperd et al. 2001), and reduction in aspen regeneration vigor

(Smith and Smith 2005), which promote conifer succession (Namroud et al. 2006). However, more research is needed to understand how gap dynamics and drier conditions projected in future climates will influence aspen–conifer succession dynamics.

4 Aspen Response to the Environment

The successional patterns of a species are partially determined by external factors (e.g., fire, climate, behavior of other organisms), as discussed above. However, the success of a species is also controlled by functional traits that determine its level of fitness in the environment. Aspen's unique characteristics have served it well under past climate conditions as evidenced by its broad distribution, but how will it respond under future climate conditions? Next, we consider how abiotic and biotic stresses influence and shape aspen fitness and consider how aspen is likely to respond to future changes in environmental conditions anticipated in its western range.

4.1 Extreme Climatic Events

4.1.1 Drought

Climate extremes are important selection forces on biological organisms (McDowell et al. 2008) and can drastically alter plant community structure and ecosystem processes (Holmgren et al. 2006; Ciais et al. 2005). In western North America, there is emerging evidence that drought events have been a principal contributor to patterns of widespread forest mortality (Breshears et al. 2005; van Mantgem et al. 2009). Research suggests aspen is particularly sensitive to water deficit (Hogg et al. 2005, 2008) and that prolonged periods of drought incite aspen dieback (Hogg et al. 2008; Grant et al. 2006). Climate projections for the twenty-first century in western North America suggest more arid conditions and increases in the frequency and intensity of drought periods (Seager et al. 2007). This suggests that mortality in western North American forests in general and aspen forest in particular will intensify in the coming century. What is lacking is a physiological characterization of the relationship between drought stress, aspen vigor, and functional traits that account for differences in drought tolerance between clones. We recently concluded a study examining variation in the functional responses and fitness of 18 unique aspen clones in a 30-year-old common garden. During the last decade of the study, there was an extended drought period. Clonal variation in photosynthesis, stomatal conductance, xylem water potential, and vertical and radial growth varied 2–3-fold. Clonal variation for canopy dieback and mortality varied 7-fold (Unpublished data). Interestingly, radial and vertical growth for all clones increased over the course of the experiment with the notable exception of a height plateau that corresponded with the drought period (Fig. 4). Canopy dieback was most prevalent at the top of the tree canopies, which would be consistent with impaired resource transport by

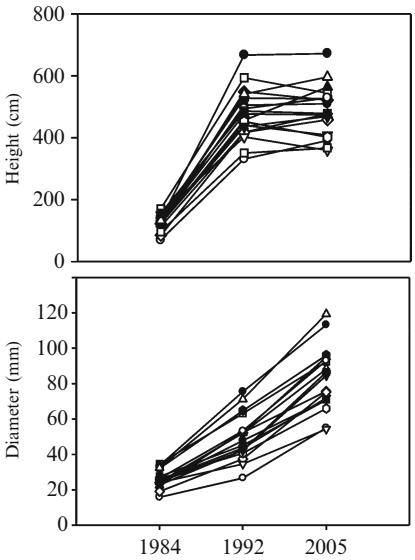

Fig. 4 A study we recently completed shows that there was no vertical growth in 18 different aspen clones during a drought period between 1992 and 2005 in a common garden study in Logan Utah. Drought conditions from 1999 to 2004 reduced water inputs into the common garden by 30%. We hypothesize that drought damaged transport tissues that carry water and nutrients to the upper canopy

vascular tissue (Unpublished data). This dieback can also be associated with Cytospora canker, which favors a drought stressed host (Guyon et al 1996).

Hydraulic limitations are known to be a key constraint to height growth in trees (Ryan and Yoder 1997; Gower et al. 1996) and an important determinant of drought sensitivity (McDowell et al. 2008). Variation in cavitation avoidance and water conservation traits may be important predictors of differential growth and vigor observed among clones in the common garden. Height growth leveled off in all clones from 1992 to 2005 demonstrating aspen's broad sensitivity to drought. There was, however, variation in height growth among clones with a few having positive height growth during the drought period (Fig. 4).

Although photosynthesis varied widely among the 18 clones, we found that xylem water potential was held within a relatively tight range of −1.5 to −2.5 MPa. Blake et al. (1996) estimated that soil moisture conditions and vapor pressure deficits that result in stem xylem pressures below −2.4 MPa, tend to trigger cavitation in aspen. In boreal systems, aspen has been shown to lower stomatal conductance in response to drought and high vapor pressure deficits (Hogg et al. 2000b; Dang et al. 1997), as an avoidance strategy for negative xylem water potentials that trigger cavitation (Sperry et al. 1994). However, prolonged decreases in stomatal conductance in response to extended hot, dry summer conditions results in less fixed carbon for growth, maintenance, and defense strategies. The challenge for aspen under prolonged periods of drought is to prevent catastrophic cavitation, while maintaining a positive carbon balance (McDowell et al. 2008).

Aspen's growth sensitivity to drought has important implications on its competitive relationship with conifers during succession. A phylogenic comparison showed that conifers in general are significantly more resistant to drought induced cavitation than angiosperms (Maherali et al. 2004; Sperry et al. 1994). Among angiosperm

families, salicaceae (aspen's family) is particularly sensitivity to drought induced cavitation (Maherali et al. 2004). When drought conditions constrain aspen's height growth, its ability to compete with conifers for light is compromised. In addition, conifer establishment has the potential to alter aspen–soil moisture relations in two ways. First, as conifers increase in density and size, they increasingly compete for soil resources including water. Second, recent evidence suggests that significantly less water reaches the forest floor under conifer stands (34–44%) in the form of winter snow compared with aspen stands, possibly because of higher rates of interception, sublimation, and/or snow redistribution (LaMalfa and Ryel 2008). Lower water inputs, coupled with greater competition can exacerbate water deficits experienced by aspen in mixed stands during periods of drought.

While we have focused our discussion of drought on tree growth and fitness, water deficit also affects regeneration capacity (Frey et al. 2003). Evidence suggests that arid conditions in western North America constrain aspen germination and seedling survival (McDonough 1979). Drought can also negatively affect aspen sucker initiation and growth (Frey et al. 2003). Drought can have similar effects on conifer seedling establishment and survival (Hogg and Schwarz 1997).

4.1.2 Low Temperature

Apart from drought, little is known about the impacts of extreme climatic events on aspen health (Frey et al. 2004). Aspen is prone to winter cavitation, which is strongly correlated with the frequency of freeze–thaw events (Sperry et al. 1994). Aspen is dependent on current-year xylem production for reversal of winter embolism (Sperry et al. 1994). Therefore, under a severe carbon limitation, it is possible that low xylem production may lead to constraints in summer water transport in aspen. Angiosperms in general, and aspen specifically, show greater sensitivity to freeze–thaw induced cavitation than conifers (Feild and Brodribb 2001; Sperry et al. 1994). Hogg et al. (2002) suggested that freeze–thaw events during a period (1983–1993) of unusually light snow cover, may have contributed to aspen dieback symptoms.

Earlier and warmer spring conditions accelerates leaf phenology, which can leave young foliage prone to late spring frost damage (Hanninen 2006; Cannell and Smith 1986). Early leaf flushing in response to warmer temperatures, followed by late spring frost events can produce extensive forest defoliation events in both subalpine and boreal forest systems (Cayford et al. 1959; Korstian 1921). In both of these studies, frost damage occurred across a wide range of tree species, but a consistent pattern emerged in which deciduous species tended to be more sensitive than evergreen species, and aspen exhibited the greatest frost sensitivity among all the tree species examined (Cayford et al. 1959; Korstian 1921). Aspen tends to reflush several weeks after frost defoliation, but second flush leaves generally have altered morphology, and significant reductions in macronutrient concentrations (Table 3). More severe frost events can cause bud damage, producing only partial canopy reflushing (50–80% permanent canopy loss is not uncommon) characterized by extremely large second flush leaves (Cayford et al. 1959; Korstian 1921). More mild frost events that do not

Table 3 Second flush leaves that emerged after frost defoliation show a ~30% reduction in macronutrients

	N%	P (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	Fe (mg/g)
First flush	3.04 ± 0.14	2.37 ± 0.11	14.5 ± 0.86	14.9 ± 1.3	2.6 ± 0.07	1.4 ± 0.01
Second flush	2.31 ± 0.06	1.94 ± 0.05	13.5 ± 0.83	10.3 ± 0.48	2.6 ± 0.14	1.0 ± 0.01
P-value	0.016	0.066	0.036	0.035	0.841	0.020

First and second flush leaf samples were collected from five mature trees (> 40-years-old) on the Fishlake National Forest in central Utah in August of 2007. One side of each tree had been frost defoliated and put on a second leaf flush while the other side had retained its first flush leaves. *P*-values were calculated based on a paired t-test. Means and standards errors are presented

result in canopy defoliation have been shown to impair photosynthetic function of aspen leaves (Lamontagne et al. 1998). Almost nothing is known about how frost defoliation events influence aspen health. Recent evidence suggests that a widespread frost defoliation event in northern Arizona in the spring of 1999 was an inciting factor in subsequent aspen dieback and mortality (Fairweather et al. 2008). Biotic defoliation events, have been linked to reductions in carbon gain (Hart et al. 2000), growth rates (Cooke and Roland 2007), and dieback symptoms (Hogg et al. 2002).

4.2 Biotic Stresses

4.2.1 Herbivory

Aspen is a primary host to well over 100 species of herbivores, including mammal, bird, and arthropod species (Lindroth and Hwang 1996a; Romme et al. 1995). Although aspen has well-developed defensive mechanisms, it is nevertheless, susceptible to a wide range of biotic stresses. When combined with abiotic stresses, such as drought, herbivory can significantly impact aspen vigor and lead to health decline (Hogg et al. 2002).

Ungulate Browsing

Experimental studies and long-term correlative studies indicate that browsing by ungulates, and elk in particular, can be a major barrier to successful aspen regeneration, (Sankey et al. 2006; Romme et al. 2005; White et al. 1998). Kay and Bartos (2000) have argued that changes in grazing pressure since the late 1800s have been the driving force behind western North American aspen decline. The impact of browsing on aspen regeneration varies greatly depending on forest management practices, habitat conditions, and climate characteristics (Romme et al. 1995). While evidence suggests that wildlife and livestock can have equally negative effects on aspen regeneration (Kay and Bartos 2000), relatively little research has been done to partition the impacts that these two groups have on regeneration failure.

Studies suggest that aspen regeneration success was relatively high at the beginning of the twentieth century due to optimal environmental conditions and

declines in ungulate populations in the late 1800s (Meagher and Houston 1998; Romme et al. 1995). Reductions in aspen regeneration during the twentieth century have been associated with increases in ungulate population sizes and changes in behavior due to predator removal (Halofsky and Ripple 2008; Ripple and Beschta 2004). While intensive livestock grazing over the last century has likely influenced regeneration failure in western aspen, few studies have examined the historical impacts of livestock grazing.

Defoliating Insects

In the upper Midwestern US and Canada, aspen experiences regular and expansive outbreaks by defoliating insects including forest tent caterpillars (FTC, *M. disstria*), gypsy moths (*Lymantria dispar*), and large aspen tortrix (Mattson et al. 1991). In the western US, several species are found to be important defoliators of aspen including western tent caterpillar (*Malacosoma californicum*), FTC, and the large aspen tortrix (Jones et al. 1985). A recent analysis suggests that projected warming trends in western North America are also likely to promote the establishment of gypsy moth populations in the western US (Logan et al. 2007). The most extensive aspen defoliator is the FTC. This native generalist defoliates an average of 936,000 ha forest in the United States each year (Mattson et al. 1991). In the Great Lakes region, where FTC is most important, populations fluctuate with approximately 7–12 year cycles (Fitzgerald 1995). Outbreak populations are typically sustained for several years. At peak population densities, FTC may cause 100% defoliation over vast reaches of aspen forest (Duncan and Hodson 1958). Severe defoliation over multiple years markedly suppresses aspen growth (Fitzgerald 1995; Churchill et al. 1964; Duncan and Hodson 1958), and moderately increases mortality in understory trees, but generally, causes limited mortality in otherwise healthy upper canopy trees (Churchill et al. 1964; Duncan and Hodson 1958). An indirect consequence of outbreaks includes increased susceptibility to Hypoxylon (Anderson and Martin 1981) and Cytospora cankers (Guyon et al. 1996), both significant causes of mortality in mature aspen trees.

Aspen's Defensive Mechanisms

The chemical ecology of aspen has been studied extensively and allelochemicals have a significant role in shaping the trophic interactions of aspen forests. Aspen produces two classes of phenolic-based allelochemicals from the shikimic acid pathway: condensed tannins and phenolic glycosides. Foliar concentrations of both classes of compounds are variable among aspen clones in the field (Donaldson and Lindroth 2008; Osier et al. 2000; Lindroth and Hwang 1996b) and in common gardens (Donaldson and Lindroth 2007; Osier and Lindroth 2001). Condensed tannins can account for 2% to almost 30% of aspen dry leaf weight (Lindroth et al. 1999; Lindroth and Hwang 1996b). In common garden studies, tannins vary

by genotype, but gradients of resource availability (e.g., light and soil fertility) and ontogenetic shifts in allocation more significantly affect foliar concentrations (Hwang and Lindroth 1997; Osier and Lindroth 2001). Phenolic glycoside concentrations can vary from 1% to 20% (Lindroth et al. 1999; Lindroth and Hwang 1996b). In contrast to tannins, phenolic glycoside concentrations show little plasticity to changing resource availability. Phenolic glycoside concentrations vary significantly by genotype and decrease as clones age (Donaldson et al. 2006).

Condensed tannins do not negatively influence aspen adapted herbivores (Donaldson and Lindroth 2007; Hwang and Lindroth 1998; Ayres et al. 1997; Bryant et al. 1987) and, their ecological significance in aspen remains unclear. In contrast, phenolic glycosides have significant biological activity against aspen adapted herbivores. For example, both field and laboratory insect performance assays have indicated that these compounds reduce growth and survivorship in generalist herbivores of aspen including the forest tent caterpillar (Osier et al. 2000; Hwang and Lindroth 1997, 1998; Lindroth and Hwang 1996a). Although most of these studies are correlative, studies that incorporate phenolic glycosides into artificial diets confirm their biological activity (Hemming and Lindroth 1995; Lindroth and Bloomer 1991; Lindroth and Hemming 1990). Wooley et al. (2008) recently demonstrated that elk preferentially consume aspen clones with lower concentrations of phenolic glycosides, which can result in selection for aspen with high levels of phenolic glycosides (Bailey et al. 2007; Erwin et al. 2001). However, when elk densities are high or forage is scarce, elk may browse even well-defended clones.

Evidence now suggests that aspen utilize different defensive strategies depending on their stage of development. In field studies, it has been found that young shoots use a strategy of "resistance" in which they produce high concentrations of phenolic glycosides early in development, and then reduce defense chemistry as they age (Donaldson et al. 2006). This pattern is likely a result of aspen's susceptibility to damage when within reach of browsing mammals or other damaging herbivores. In contrast, mature canopy-dominant aspen are likely adapted to the cyclical nature of defoliating insect outbreaks, during which insect populations become so dense that all clones, regardless of allocation to defense, are defoliated. In such a scenario, rather than investing significantly in defense compounds, "tolerance" is likely to be the more favorable strategy. Tolerance is defined by a species ability to compensate for damage or lost tissues, and aspen does exhibit high levels of tolerance to herbivore damage (Stevens et al. 2007). Aspen's clonal habit, and high stem and root to leaf ratios allow for substantial storage of carbohydrate and nutrient reserves that allows for compensatory growth following defoliation. In addition, partial defoliation in aspen can lead to increased penetration of sunlight into the canopy and increased photosynthetic efficiency of lower canopy leaves. Defoliation also leads to increased soil water availability (decreased transpiration) and photosynthesis rates (physiological response to defoliation), which can compensate for defoliation losses (Kruger et al. 1998). Aspen's clonal nature and extensive root and stem system are important traits that allow it to persist until it is released from intensive herbivore pressure.

4.2.2 Diseases and Stem Insects

Aspen plays host to a large number of insect and disease causing agents (Hinds 1985; Jones et al. 1985), the impacts of which are often poorly understood. Additionally, the suite of damaging agents present change as clones age, with increasing biotic or abiotic stress, and degree of wounding.

Foliar Diseases

Many diseases are commonly found on aspen foliage, but only a few are seriously damaging. Marssonina leaf blight, caused by *Marssonina brunnea* (Ell. and Ev.) Magnus and *Marssonina populi* (Lib.) Magnus (Sinclair and Lyon 2005), is the most common and damaging foliar leaf blight of aspen. This disease has been known to defoliate entire clones over broad landscapes in some cases (Harniss and Nelson 1984). Venturia shoot blight, caused by *Venturia tremulae* Aderh., causes a blight of young succulent stems in moist conditions (Sinclair and Lyon 2005). This disease is important in young ramets, sometimes damaging or killing terminals and, in some cases, causing severe reduction in height growth (Peterson and Peterson 1992). When this disease is common, young ramets can have a zigzag appearance due to repeated death of the terminal. Viruses, such as the poplar potyvirus, also have the potential to be significant factors in a declining forest. This virus is common in the Lakes states (Martin et al 1982), but its symptoms have not as yet been associated with the current decline syndrome.

Root Diseases

Armillaria root disease in aspen is widespread and associated with declining forests in both western and eastern North America (Brandt et al. 2003; Hinds 1985). *Armillaria* spp. have a wide host range, and the degree of pathogenicity of Armillaria on aspen is largely, uncharacterized but it is known to infect wounds caused by mechanical site preparation (Pankuch et al. 2003) and is sometimes seen killing aspen closely associated with conifers in the Rocky Mountain and Intermountain Regions (Worrall et al. 2004; James and Goheen 1981). Ganoderma root disease is commonly associated with aging ramets in the Intermountain Region (Krebill 1972), causing windthrow and contributing to the breakup of aging stands.

Agents Attacking the Stem

Two of the main groups of agents attacking the stem of aspen ramets are wood-boring insects and canker diseases. Two primary wood borers are commonly associated with the decline in the Rocky Mountain and Intermountain Regions; poplar borer and bronze poplar borer (Worrall et al. 2008; Guyon 1998). Both of

these agents are primary considered to be agents of stressed trees (Ostry et al. 1988), but they seem to be acting more aggressively as part of the aspen decline scenario. Another stem boring insect, a clear wing borer *Paranthrene robiniae* (*Henry Edwards*) is important in the aspen decline in Arizona (Fairweather and Worrall 2007 The aspen bark beetles, have played a similar role to stem borers in widespread aspen mortality in Colorado (Worrall et al. 2008). In addition to the two primary canker diseases described above, Ceratocystis canker (*Ceratocystis fimbriata* (Ellis and Halst.) Sacc.) is also common, but it is a slow moving disease and does not appear to be increasing in prevalence or damage with the decline. Hypoxylon canker is the most important canker disease in Great Lake states forests (Ostry et al. 1988), but is much less common and damaging in the west. Canker diseases usually require wounds created by to initiate disease, animal damage due to bark striping, barking, and antler rubbing. Browsing is a common source of wounds in aspen (Debyle 1985), (Jones et al. 1985). Hart (2001) noted that mortality rates nearly double in all size classes, when heavy elk use was present largely due to wounds becoming infected by canker diseases. The most interesting canker/host relationship in our survey occurred with *Cytospora* canker. It has been observed when *Cytospora* moves out of its usual role as an agent of minor branch dieback and a killer of small stressed trees that a clone is under significant environmental stress.

Over 250 fungi are capable of decaying the stem and roots of living aspen, but only a few are common and damaging enough to play a role in aspen decline. The most common and damaging stem decay organism on aspen is white trunk rot (*Phellinus tremulae* (Bondartsev)). This disease is thought to control stand rotation age in aspen (Jones and Shepperd 1985), which is the age at which the rate of accumulated aboveground stand woody biomass is eclipsed by the rate of decay and stain in the wood. This fungus is usually involved in the gradual break up of older stands where decay has had time to develop, but can appear in younger stands that have high levels of wounding (Hinds 1985). Two other fungi, *Peniophora polygonia* (Pers.:Fr.) Bourd. and Galzin, and *Cryptosphaeria ligniota* (Fr.) Auersw., which also causes a canker disease, are very common decayers of aspen, but their role in stand decline is unknown (Hinds 1985).

5 Mechanisms Underlying Decline

The decline concept can be useful in describing aspen decline symptoms and the role of abiotic and biotic stresses (Guyon 2006; Frey et al. 2004). As defined by Manion (1991), a decline refers to gradual deterioration in the health of many individual trees in a population leading to mortality. In this definition, the disease typically has the following traits (Worrall et al. 2008): (a) gradual loss of vigor and eventual death of large numbers of trees; (b) affects primarily the most mature component of a population, often the dominant and codominant trees; (c) feeder roots and mycorrhizae degenerate prior to onset of symptoms in the aboveground

portion of the tree; (d) depletion of stored carbohydrate reserves; (e) various symptoms of poor growth and low vigor; (f) caused by three types of factors: predisposing factors are long-term, slowly changing factors, such as age, soil conditions, and long-term climate changes. Inciting factors are short-term physiological or biological factors that cause brief, acute stress, including insect defoliation, extreme climatic events, and air pollution. With these factors alone, trees may recover quickly, but recovery is much slower if the population is affected by predisposing factors. Finally, contributing factors kill trees that have been debilitated by predisposing and inciting factors. Contributing factors are mostly biological agents, including canker fungi, wood-boring insects, and bark beetles. The contributing factors are usually most apparent and are often blamed for the decline.

Exposures to predisposing and inciting factors deplete aspen's carbohydrate reserves (Landhausser and Lieffers 2002), which are needed to fuel growth for additional light and soil resource capture. Furthermore, carbohydrates are the starting material for producing defense compounds to deter herbivores. Thus, aspen faces a dilemma: under conditions that shrink its carbohydrate reserves, how does it simultaneously maintain rates of growth and effectively defend itself (Herms and Mattson 1992)? Conifer encroachment and lengthening disturbance cycles require aspen to grow as rapidly as possible to compete with conifers for resources it needs to persist in the landscape. However, carbon rich defense compounds are also needed to defend aspen from herbivores and pathogens. Clearly a threshold point can be reached in, which depleted carbohydrate pools are insufficient to maintain adequate rates of growth and/or produce a robust defense strategy, which then reduce aspen's fitness (Fig. 5).

6 Aspen's Clonal Nature

Aspen's clonal habit is a conspicuous and critical feature influencing its ecology. The large size and the persistence of single aspen clones over presumably thousands of years, in environments that have experienced large fluctuations in climate conditions, attest to the robustness of aspen's clonal strategy. The rapid and vigorous regeneration following disturbance events allows aspen to capture resources that are readily available following disturbance events. Survival of roots following shoot removal or dieback not only allows the clone to survive but results in an adjustment in the root to shoot ratio that may be more adaptive (e.g., drier conditions). The large size of clones also provides some buffering capacity. For example, the vast carbohydrate storage capacity and allocation that can occur within an aspen clone may provide it with the resources and flexibility needed to adjust and persist during extended periods of stress.

The current paradigm of aspen regeneration in the Rocky Mountains is that it occurs almost exclusively through clonal reproduction, because it is assumed that moisture conditions are not optimal for seed germination and establishment

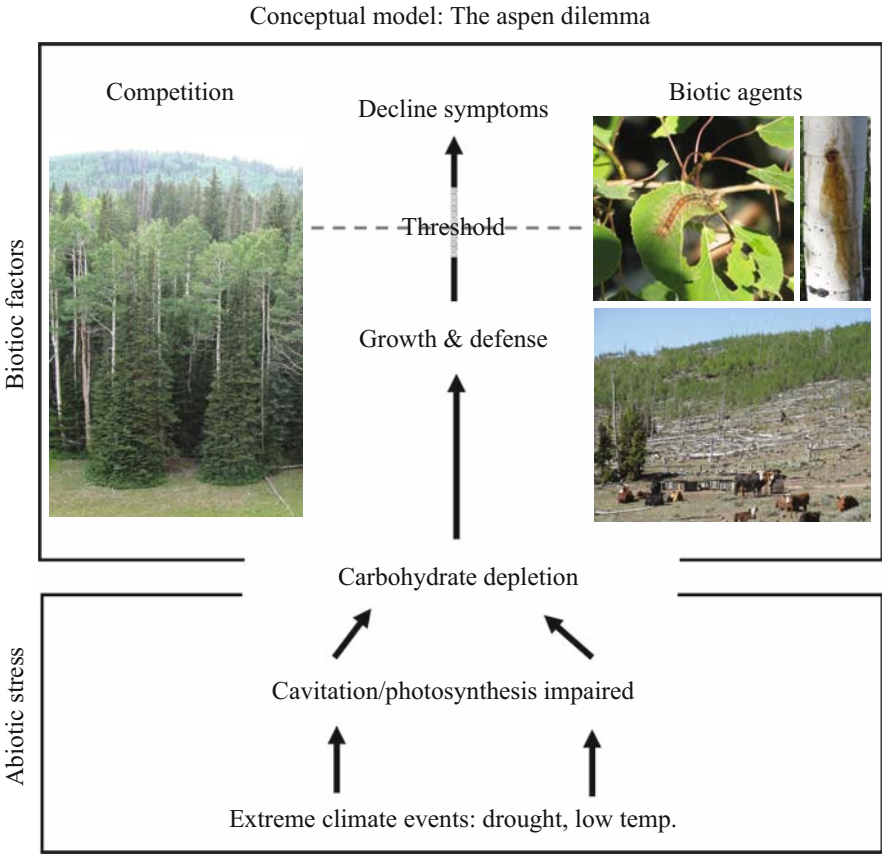

Fig. 5 Conceptual model outlining the mechanisms that lead to aspen decline. Abiotic stresses deplete carbohydrate reserves that are necessary for growth and defense. If severe enough a threshold is reached in which carbohydrate depletion constrains growth and/or results in inadequate defense resulting in reduced fitness that leads to health decline

(McDonough 1979). However, recent genetic studies suggest that aspen seedling establishment may be more common in its western range than previously thought (Mock et al. 2008) and may correspond to episodic events linked to certain environmental conditions (Romme et al. 2005). Thus, seedling establishment by aspen may prove to be an important source of genetic diversity for western aspen populations. However, because of the prevalence of clonal regeneration in aspen's western range, its influence on aspen ecology cannot be understated. Clonal regeneration is not a source of genetic variation and is slow as a dispersal mechanism for adapted genotypes. Because ramets within a clone are genetically identical, selection forces can impact entire clones. The success of aspen across the diverse environmental gradients of western North America and in the face of climate change is, therefore, dependent on phenotypic diversity that exists between clones.

This may be even more important in the future if drier conditions place even greater constraints on seed germination and establishment.

Phenotypic diversity in aspens is among the largest observed in tree species (Barnes 1975). Significant trait variation between clones is observed in growth (Oksanen et al. 2001), leaf morphology (Kanaga et al. 2008; Barnes 1975), foliar chemistry (Hwang and Lindroth 1997; Lindroth and Hwang 1996a), and phenology (Yu et al. 2001). Variation in stress tolerance accounts for differential clone success in response to selection forces, such as ozone (Oksanen et al. 2001) and drought (Griffin et al. 1991). A nationwide (US) survey of aspen populations along a gradient of ozone concentrations showed that aspen sensitivity to tropospheric ozone was inversely correlated ($r = 0.925$) with ozone concentrations from which the populations were collected (Berrang et al. 1991). These results suggest that ozone is an important selection force on aspen functional trait evolution. It is likely that other environmental factors, such as drought and temperature stress also exert strong selection influences on aspen across its wide geographic and climatic range. Physiological tradeoffs under variable environmental conditions may explain why large genetic variation is maintained in species with broad environmental ranges, such as aspen (Donaldson et al. 2006).

In addition to the phenotypic variation that exists between clones, there is also ontogenetic trait variation within clones that likely contributes to aspen's success. A characteristic feature of stable aspen stands is that they self-regenerate (in the absence of disturbance), resulting in mixed age stands (Kurzel et al. 2007). Current research demonstrates that phenotypic traits in aspen are highly variable among the different age cohorts (Donaldson et al. 2006). Thus, within a single genetically identical clone, the multiple age classes provide additional phenotypic variation that increases the likelihood of clone survival in response to shifts in selection pressure.

7 Conclusions

In summary, there is spatial variability in aspen vigor across its western range. In some regions, there do appear to be trends of canopy dieback and habitat loss but further research is needed to determine the scale and magnitude of decline events and whether they are beyond historical limits. Several environmental factors appear to be having important influences on aspen's vigor and distribution. Longer fire return intervals promote conifer establishment, which alters soil and light conditions that reduces aspen vigor and regeneration success (Smith and Smith 2005), and promotes aspen succession to conifer (Namroud et al. 2006). However, more research is needed to understand how gap dynamics and drier conditions projected in future climates will influence aspen–conifer succession dynamics. Extreme climatic events, such as drought and freeze–thaw cycles that are projected to intensify under future climate scenarios are anticipated to reduce aspen vigor through cavitation, reductions in carbon gain, and

defoliation events. Competition with conifers and greater exposure to extreme climatic events can be expected to place constraints on physiological function resulting in carbon depletion that will compromise defense strategies against herbivores and pathogens. Additionally, warmer conditions may favor establishment of insect defoliators, which are often limited by minimum winter temperatures. Changes in aspen phenology in response to a warmer climate could make aspen more or less susceptible to both insects and fungal diseases. Drought events and other environmental extremes can act as inciting factors leading higher levels of biotic stress. More research needs to be done to determine the frequency of sexual regeneration in aspen's western range. While clonal regeneration is not a source of genetic variation in aspen, inherently large phenotypic variation between clones and within age classes of clones does provide trait variation critical for maintenance of aspen in changing environments. However, if seed regeneration is limited, dispersal of adapted genotypes may be a critical limitation to aspen maintaining its expansive and dominant position in western subalpine landscapes.

Because of aspen's keystone role in both boreal and subalpine forest, changes in its presence or abundance in these systems will have effects on the ecosystem services they provide. Potential consequences include loss in biodiversity, reductions in climate and CO_2 buffering capacity, and lower water yields out of western mountain watersheds.

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Quaternary Palaeoecology of East and Southeast Asia and of the Pacific Ocean

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Contents

Abstract The base for palaeoclimatological considerations is remarkably rich in unproven hypotheses, because the interests focus on relatively easily understandable major problems, but not on the wealth of involved facts, particularly regarding regional aspects. Further, the vegetation reconstruction of former ecotones by the analysis of small-scale modern pollen sedimentation-rates of certain vegetation types bears serious methodological difficulties. Regarding the reconstruction of the Late-glacial and Holocene forest immigration processes on the Tibetan Plateau, major steps of the vegetation history and their timing are already quite clear, while the composition of the involved plant associations and the related basic palaeoecological processes are still poorly understood. Despite of the existing, most interesting, and well-dated work, this is similar for the Upper-Quaternary vegetation history of north-eastern Asia. Like for other glacial refuge areas, the question, where glacial forest-refuge areas might have been situated and how their plant communities might have been composed, remains mostly unanswered. Even more problematic are the immigration of *Homo sapiens sapiens* to Korea and some of the Japanese Islands (at about 40,000–30,000 BP) and the related palaeoecological consequences. Interestingly, the island of Ryukyu seems to have been settled at about 30,000 BP by people coming from much farther south, when sea-level was low. Regarding man's settlement in SE-Australia and the surrounding islands

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(at about 43,100 ± 6,700–34,000 ± 3,900 BP), it is still mostly unknown, where man came from, although abundant data material on his impact on the vegetation (particularly from Papua New Guinea) is available.

1 Various Problems of the Reconstruction of Palaeoclimates

Modeling of past and future climates has become an important tool for evaluating future and past tendencies in the ecosystem development and thus, for understanding the reasons for local and regional changes, which repeatedly happen in the ecosphere. Li et al. (2007b) developed a transfer-function model for understanding the dependence of North Chinese ecosystems on climate change. They used 237 soil and surface pollen-spectra of "less disturbed" (what does this mean?) plant communities of northern China and stated that their method works quite well at an annual precipitation-rate between 40 and 800 mm and at mean annual temperatures between −4° and ca. +13°C. However, the ecology commission of the Bavarian Academy of Sciences (Munich, Germany) very critically analyzed the methods, presently applied in climate modeling. According to Graßl (2005), seven different types of pluviometers, which are presently used on the Isle of Fehmarn, differ in their results during rainy weather by a factor of 2, and when in the case of snow by even more. Further, it seems that the full-glacial atmospheric CO_2-values of 190–200 ppm and of 280–290 ppm during interglacial times are still not really understood. Volz (2005) pointed to the fact that the emissivity (i.e., the irradiance of heat) of the ocean's surfaces is not correctly taken into consideration when the global heat balances of various times are being analyzed. This is particularly true for full-glacial times when wind speeds had seriously increased in comparison to the modern situation. Thus, the author states that for analyzing full-glacial climate conditions, the atmospheric circulation patterns and their strengths would be of much greater importance than the oceanic thermohaline circulation, which is always solely taken into consideration. Fiedler (2005), when discussing climate history, states that in contrast to the influence of radiation, the amounts and significances of evaporation and of turbulent heat-transports are not properly taken into consideration, although these factors contribute approximately 2/3 to the cooling of the earth's surface. Furthermore, the global precipitation values are still poorly understood. Nowadays, the CO_2-contents of the Earth's atmosphere are being intensively discussed. However, a doubling of the atmospheric CO_2-concentration would affect the atmosphere's energy fluxes only by about 1–3 W m^{-2}, while our planet receives 341 W m^{-2}. Thus, the problem is, whether such small changes can be correctly quantified. Another climatologically important factor is the aerosol-contents. According to Heintzenberg (2005), the magnitudes and standard-deviations of these contents in the recent atmosphere are only poorly understood even today, although the drought of the Sahel-zone had strongly influenced these values during the last 30 years. Egger (2005) points out that grid point distances of about 100 km are generally being used for climate modeling,

although distances of 10 km would be much more appropriate for regional climate models. Finally, Solanki (2005) discusses various aspects of solar influence on global climate in this connection.

In general, *Sphagnum*-mosses are widely used in palaeoecological research. For analyzing seasonal changes of pollen-influxes to these mosses, Joosten and de Klerk (2007) analyzed 0.5 mm thick slices of *Sphagnum cuspidatum*. However, this did not work due to small changes in water-levels before pollen-grains had been definitely absorbed and because *Sphagnum*-branches often grow only horizontally. A key for the determination of brown-moss species of peats by their leaflets can be found in Michaelis (2001).

Loader et al. (2007) investigated the $\delta^{13}C$-values of different organs of two *Sphagnum* species from alpine region of Lungau, Austria. Evidently the results are good, if isotopic differences in the contents of various organs are properly taken into consideration. The base for all palaeoclimatic analyses using isotope contents is a good knowledge of these contents in precipitation and seawater. This has been analyzed by Tyler et al. (2007) for eastern Scotland. Quite another approach was the thickness measurements of aragonite-layers from Madagassian stalagmites for studying precipitation and temperatures, prevailing during the warm season (Brook et al. (1999). However, February and Stock (1999) could not find any correlation between the $^{13}C/^{12}C$-ratios of *Widdringtonia* tree-rings and the amounts of precipitation in southern Africa. Regrettably, the correlation with temperatures was not analyzed. Eggerment et al. (2006), based a study of past salinities of east-African lakes on the occurrence of various *Chironomidae*-species at certain times. They stress that there is no simple statistical procedure for determining the past salinities and it is, therefore, necessary to study the ecology of past *Chironomidae*-societies in total. Comparable difficulties are described by Gasse et al. (1995) on one hand, who deduced the former chemistry of east-African lakes from the diatom communities in the lake-sediments. On the other hand, Trauth et al. (2006) found anticorrelations between lake-levels of east-African lakes (within the tectonic zone) and the palaeoclimate of west- and northeast-African regions.

Human impact has always been a very important palaeoecological factor among most of all, as climate is concerned. Trace-gases play an important role in this connection. According to Saarnak (2001), man-made fires in the savannah-zone were obviously very important resulting in a threefold increase of trace-gases compared to conditions unaffected by man. Similar results have been shown by Girardin and Sauchyn (2008) for north-western North America and by Duffin et al. (2008) for the Krüger National Park, South Africa.

Mahaney (1995) tried to determine pleistocene and holocene glacier thicknesses, transport histories, and dynamics by analyzing the SEM microtextures of quartz grain particles in tills of various regions of the globe. Yet to my own experience, this seems to be impossible. However, Imaeva et al. (2007) analyzed geological structures of the Upper-Quaternary, together with traces of recent geodynamics of the Arctic sector within the shelf-region of the northern Verkhoyansk area of easternmost Yakoutia, presenting interesting maps of earth-quake frequencies and of tectonic movements.

2 Attempts to Reconstruct Palaeovegetation Correctly

Charred material of small seeds of certain legumes is frequently found in epipalaeolithic, prepottery, and neolithic layers of southwestern Asia, of northern Africa and of southern Europe. Butler (1995) analyzed seeds of *Trifolium, Trigonella, Medicago*, and *Melilotus* by means of written reports, archeological, ethnographical, and biochemical descriptions. It turns out that these seeds had been used in great quantities, perhaps in strongly grazed localities, and as protein- and oil-resources as well. Bieniek (1999) studied the contents of three wells at Wąsosz Górny (Central Poland), which date from Roman Iron Ages. The author tries to correlate certain types of cultivated plants with the associated soil-types. For the first time, he found in Poland *Chenopodium ficifolium, Dianthus armeria*, *Fragaria vesca, Verbena officinalis, Centaurea phrygia*/*C.stoebe*, and *Trifolium arvense*/*T. dubium.* von Stedingk et al. (2008) studied the old problem of how to reconstruct the former vegetation at the forest – tundra ecotone, if only surface pollen-samples are available. For this, they determined pollen productivity estimates (PPEs) and the relevant source areas of pollen (RSAP). It turned out that the RSAP was generally only ca. 500 m in diameter and that the PPEs strongly depended on local situations. But, there was always a remarkable background-noise of the strong long-distance transport (ca. 60% of the pollen sum found). To reconstruct vegetation by means of pollen analyses, Sugita (2007a, b)developed two statistical procedures only based on the pollen-sums of *Poaceae, Picea, Betula*, *Quercus*, and *Fagus*. I doubt that this will suffice and this is supported by observations of Kvavadze (2000) on recent pollen-fluxes of various plant species in the Chernogora (Ukrainian Carpathians), where the maximum wind-transport of *Fagus* pollen-grains amounts to approximately 58%. Beer et al. (2007),however, investigated the pollen representation in surface samples of *Juniperus,* and *Picea* forests in Kirgizia, Central Asia. They analyzed surface samples at five localities, situated to the south of Issyk Kul. In surface areas of 10×10 m^2, the coverage of the taxa was compared to the amounts of the pollen-grains of the taxa. Within these surface areas, the correlation coefficient for *Juniperus* was 0.76, for *Picea* 0.85, and for *Juglans* 0.32. However, if areas of 800 m in diameter were analyzed, it turned out that the arboreal pollen values were less than 60%, sometimes even down to 30%, with a strong preponderance of the steppe-plants *Artemisia, Chenopodiaceae*, and *Poaceae*.

3 Upper-Quaternary Palaeoecology of Central and Southern Asia

At present, one of the main palaeoecological problems on the Tibetan Plateau is the question, to which extent today's widely distributed steppe-like vegetation in the drier parts of the plateau might have originated from climatic factors or – however – from man's direct or indirect activities. This problem has already intensively been discussed by Frenzel (2002) and authors favoring either view are

cited. Further it was discussed, whether during stadials of the Pleistocene, the Tibetan Plateau had been covered by huge inland-ice masses or only by smaller mountain-glaciations. Today, this situation has considerably changed. Based on own observations and several literature–data, Frenzel and Liu (2001) have shown, that at least during the two last glaciations no inland-ice but only smaller mountain-glaciations, had existed on the plateau. This view is supported by Kaiser et al. (2003, 2008), and by Frenzel (2006). For the Holocene history of glacial advances and retreats in this area, see Kaiser et al. (2007) and Miehe et al. (2008).

The Holocene immigration history of forests to various parts of the eastern Tibetan Plateau could tentatively by dated in several regions, too. Due to our pollen analytical work (Adamczyk, unpublished work), this seems to have happened in western Sichuan and in eastern Tibet before 9,200 to 9,100 BP (uncalibrated data), already. Here, the deeply incised valleys were obviously important immigration pathways. At the upper Mekong valley (31°N, 97°E), this seems to have happened at about 7,000 BP; on the eastern beaches of Nam Co at about 6,000 BP, and on the northern flank of Qomolongma (Mt. Everest) at ca. 5,500 BP, only. Kaiser et al. (2007, 2008) and Miehe et al. (2008) repeatedly give earlier data; however, they investigated macrofossils of trees and not the pollen-flora of certain vegetation types, as we had done.

Another still remaining problem is the timing of the first human impact on the east-Tibetan vegetation. The former state of the art can be found in Frenzel (2002). The general view (except for some Chinese papers) was, that man and his animals had intensively started to influence and change vegetation at about 2,500–800 BP. This view was not supported by Thelaus (1992), nor – quite independently – by Frenzel (1992), who reconstructed for north-western Sichuan traces of the earliest remarkable impact of man on vegetation at about 5,500 and 4,500 BP, already. It is interesting to note that Schlütz and Lehmkuhl (2009), mostly using the pollen-data of Schlütz (1999), arrive at the same conclusions as Thelaus and Frenzel. The quality of ^{14}C-datings is doubtful, because on large parts of the Tibetan Plateau permafrost causes slidings of peat layers, so that it seems to be impossible to extrapolate age-data from a few analyzed horizons to whole peat-profiles without serious controls (Frenzel 2007).

The Upper-Holocene history of certain Tibetan glaciers has been intensively analyzed based on dendrochronological investigations of Bräuning (2006, 2007), Bräuning and Grießinger (2006), Yang et al. (2008), and Wang et al. (2008). As a result, a well-dated data-base for comparisons with other mountain-regions of the globe is now available. Concerning the Late-Holocene climate history of southern India, see Gunnell et al. (2007) and Prasad et al. (2007). According to Maloney (1995), the Younger *Dryas* cooling of climate can at present not be traced in South East Asia, but it seems that during the Last Glacial Maximum, the present-day mountain forests composed of *Dacrycarpus, Podocarpus, Engelhardtia, Myrsine*, and *Ericaceae* had been depressed by about 2,000 m in Central Sumatra and western Java.

Caner et al. (2007) investigated δ^{13}C-values of organic material at various soil-depths under the “spontaneous” vegetation of the Nilgiri Highlands (Southern

India; does a spontaneous vegetation still exist there today?). These values were used for reconstructing the former distribution patterns of forests, since about 1,800 BP within the area of the Central, Western, and Southern Nilgiris. Unfortunately, the geochemical analyses are not paralleled by palaeobotanical investigations on the vegetation history of this most interesting region.

4 Upper-Quaternary Palaeoecology of Various Parts of Eastern Asia

Several problems are most interesting here, as e.g., the Upper-Quaternary vegetation history, the immigration pattern of *Homo sapiens sapiens*, and the history of agriculture, most of all that of the cultivation of rice.

Kuz'min (2004) very comprehensively analyzed the Upper-Quaternary palaeoecological development of the Russian Far East, based on at least 43 ^{14}C-datations (map of the full-glacial vegetation of the area studied). Coniferous and broadleaved forests were widely distributed at about more than 38,000 to > 40,000 years BP in the southernmost part of this vast region (Peščera Geografičeskogo Obščestva, map). They may date from interstadial or even interglacial times. However, a full-glacial vegetation was present in the very vicinity of this interesting site at about 20,000–18,000 BP, which was replaced at about 15,900–15,100 BP by *Betula–Corylus* forests, which also occurred in other regions. At about 11,800–11,600 BP, this vegetation was enriched by the first broadleaved trees, together with *Corylus*. At about 10,800–9,500 BP, neolithic man (ceramics!) seems to have lived in this vegetation, but he was found there during full-glacial times already. The full-glacial conditions at Ust' Il'ma, near to the large bend of the river Zeya (about 20,000–19,000 BP), when permafrost still had been the decisive ecological factor, were already replaced at about 14,200–10,500 BP by *Betula–Larix* forests, into which *Quercus*, *Ulmus*, and *Tilia* were immigrating. Kuz'min et al. (1998) analyzed the development of man's activities within the Russian Far East based on 150 ^{14}C-data. Microblade technology had been used there since about 20,000 BP. This ended at different times depending on the region of concern, i.e., along the Middle Amur river about 10,500 BP and in the Primor'e at ca. 7,800 BP, first signs of marine fishing (shell-fish) occur at about 6,400 BP and agriculture seems to have emerged in the Primor'e since about 4,200–3,700 BP.

Comparable investigations were carried out by Bae (2002) in Korea. At that time, only less than 20 ^{14}C-datations were available for the Korean paleolithic. The lower boundary of the Upper Paleolithic seems to date there from about 30,000 BP. Lim et al. (2007) described changing palaeoecological conditions in Gwangju, Southwest Korea. The geological sequence (fluvial sands, mud and mud penetrated by roots) seems to be disturbed. The pollen-analytical work shows the retreat of *Quercus lepidobalanus*, together with a certain increase in *Castanopsis* and of *Gramineae*. It is assumed that the small increase in NAP at about 3,300–2,600 BP had been caused by climate change, although the activities of man are not properly considered.

Li et al. (2007a) analyzed the history of early agriculture within the basin of Tian shui (about 34° 54′ N, 105° 33′ E, 1,330 m asl). This is an area, which was touched by the famous silk-road. It is said that the 650 cm thick geological profile would be undisturbed, but the eight available ^{14}C-data are completely in disorder. The profile begins at about 5,035–5,295 ca. BP. It is said that nearly from the beginning onwards *Oryza sativa, Setaria italica, Panicum miliaceum,* and *Avena* sp. had been present. Later, the proportion of *Picea*/*Abies* and of *Pinus* rapidly decreased, whereas *Castanea, Polygonaceae,* and *Artemisia* drastically increased. Zhao et al. (2007) studied the loess and palaeosoil-sequence 120 Km to the north of Xi'an. The sequence begins at about 18,240 and 23,710 BP, yet the base of the first Holocene palaeosoil dates from 8,420 ± 1,620 BP. The formation of this soil ended at about 3,700 BP, followed by two younger soils and loess-layers. It is assumed that the soil dates from the Holocene climatic optimum. Selivanov (2004), using Chinese literature, has mapped the configuration of Bo Hai, since about 114,000–20,000 BP, together with the courses of Hoang Ho and Huai Ho. Dearing (2008) investigated the evolution of the Yunnan-landscape during the last 3,000 years, stressing – quite understandable – that during this time, the impact of man has been much more important there than the influence of climate.

An important question of East Asian palaeoecology is, when the cultivation of rice may have begun. This has already been discussed by Frenzel (2002). Liu et al. (2007) again stress this problem, based on a map of the 21 oldest finds of rice in East Asia. It is argued that the oldest finds originate in the areas of the lower Yang tse river and of the Huai Ho (at about 9,000 BP). Of course, wild and, to some extent, already cultivated rice were used simultaneously in these oldest times. These age-data were confirmed by Atahan et al. (2007). Evidently, traces of the oldest agriculture in the valleys of the Hoang Ho and of the Yang tse jiang date from about 9,000 cal. BP. It can be shown that *Oryza sativa* had developed from *O. rufipogon*, which had spontaneously been distributed during the Holocene climatic optimum up to the valley of Huai Ho. It seems that from here the cultivation of rice expanded to Thailand (4,300 cal. BP) and at about 4,000 to Malaysia and to the Philippine Islands. At about 4,600 BP, it had already been intensively cultivated within the valley of the Indus river, and at about 5,000 BP in the Ganges valley. For the much younger human impact on vegetation in the surroundings of Shang Hai, see Itzstein-Davey et al. (2007a, 2007b).

According to Takamiya and Obata (2002), the Japanese Islands seem to have been settled by *Homo sapiens sapiens* since about 30,000 BP, i.e., by people, whose cultural traditions point to that of Korea. However, on Ryukyu, several human skeletons (Minatogawa) point to people, that had come from southern China (man of Liujin), but which do not show connections to the man of Zhoukoudian in northern China. Thus, it seems that Central Ryukyu has been populated by people, who had come from the south, perhaps from Taiwan or even from farther to the south, when sea-level stood low. Since 30,000 BP, Kyushu and Shikoku were settled uninterruptedly, while on Ryukyu, settlement seems to have been reduced or even interrupted from the end of the Pleistocene, up to ca. 3,000 BP. For more details on the timing of Japanese settlements, see Ono et al. (2002).

5 Quaternary Palaeoecology of the Southern and Central Pacific Ocean

Vegetation history and human impact on the Quaternary palaeoecology of the southern and central Pacific Ocean has repeatedly been analyzed in Australia. Here, the oldest traces of human activities (Lake Mungo, SE-Australia) seem to date from about 43,100 ± 6,700 and 34,000 ± 3,900 BP (Oyston 1996). Regrettably, the "species" of man responsible is unknown. In New South Wales, Australia, the earliest impact of man (initial habitation) seems to date from about 22,400 ± 1,000 BP, when charcoal layers were formed repeatedly (Black et al. 2008). It is interesting to note that within the Little Desert National Park, Victoria, Australia, since at least 9,000 BP till ca. 4,000 BP, the prevailing vegetation dominated by *Allocasuarina* spp., *Callitris* spp., and *Gyrostremonaceae* was replaced since ca. 4,000 BP by *Poaceae, Asteraceae, Chenopodiaceae, Restionaceae,* and by two types of *Allocasuarina*. It is shown that fire-frequency had increased simultaneously, but it is unknown, whether this was due to climate change or to the activity of man (Thomas et al. 2001). Comparable questions were analyzed in central Australia by Bowman et al. (2007) concerning the *Acacia aneura–Triodia* boundary. The site is situated at the southern boundary of the Tanami-Desert, where groves of *Acacia* in *Triodia*-steppes are characteristic elements of the dominating vegetation. Although 16 AMS-dated soil-profiles were analyzed ($\delta^{13}C$, $\delta^{15}N$), it remains unknown which factors had definitely influenced the position of the borders of *Acacia* groves against those of the steppe.

Within the Australian drylands, the history of man can be followed since about 19,000 cal. BP (Smith et al. 2008). Obviously, there are no clear connections between environmental changes and the different phases of human activities (Smith and Ross 2008). This was confirmed by Denham and Mooney (2008). The same holds for the comparison of phases of increased production in the amounts of charcoal layers and layers of artifacts (Black et al. 2007). Cohen and Nansen (2007) point to the fact that in south-eastern Australia, the ecological development of large lowland valleys differed remarkably from the more or less narrower upland swamps. It seems that in areas of higher elevation, the vegetation repeatedly changed during the Holocene and it is therefore difficult to compare both these types of landscape with one another (Nott et al. 1999). Moreover, it should be taken into consideration that at least in South Australia, the impact of the indigenous human population strongly differed from that of the European immigrants: Although the indigenous population had intensively affected the vegetation growing close to the soil, it had no major influence on the higher growing tree-layers (Bickford et al. 2008). Regarding the influence of marine transgressions on the Australian coastal vegetation, see Rowe (2007) and Switzer and Jones (2008). For the Upper-Quaternary history of Australian lake-level changes, see Harrison (1993); however, in my opinion, Mrs. Harrison has by far over-interpreted the available data.

It is interesting to note that on Papua New Guinea (Willaumez Peninsula), the oldest dates of human influences on landscape date from about 40,000 BP.

It is stressed by Petrie and Torrence (2008) that the several volcanic eruptions did not seriously affect the human activities there. This even holds for the cultural development. Denham and Haberle (2008) intensively discussed the history of agriculture and the anthropogene transformation of various landscape types within the upper Wahgi valley, central Papua New Guinea mountain region. Of course, the reasons for fire at about 35,000–27,000 BP are difficult to trace there, when precipitation seems to have been high. During the Last Glacial Maximum, temperatures seem to have decreased there in comparison to modern conditions by about 5–7°C. Moreover, dry periods could be traced for this region at about 27,000–24,000 BP, 18,000–11,000 BP and from 6,000 BP to modern times. From about earlier than 32,000–21,000 BP, valley-floors seem to have been covered by a mixed mountain forest, in which human disturbances could not be found at all. But human disturbances occurred from 21,000 to 7,000 BP, when a secondary vegetation spread, characterized by *Trema*, *Acalypha, Macaranga,* and *Dodonaea*, together with several indicators of fire. During the last 10,000 years, man's influence became first evident at the forest borders: Grassland expanded, however the settlements were used for only short times. From 17,000 to 11,000 BP, forest clearances had already been practiced in the upper Wahgi valley and *Musa* spp., *Saccharum* and *Setaria palmifolia* were increasingly planted. However, David (2008) very critically discussed 100 AMS-^{14}C-data available from the Kopi region, Gulf Province, Papua New Guinea. It becomes evident that from 13,000 to 8,000 cal. BP, only faint traces of human activities could be found there, while the majority of them begins at about 4,000 BP. Summing up this entire recent information, one can now much better understand what might have happened there during Upper-Quaternary than this was possible only some years ago.

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