Sclerotial metamorphosis in filamentous fungi is induced by oxidative stress

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Synopsis Sclerotium-forming filamentous fungi are of great agricultural and biological interest because they can be viewed as models of simple metamorphosis. They differentiate by asexually producing sclerotia but the processes involved in sclerotial metamorphosis were poorly understood. In 1997, it was shown for the first time that the sclerotial differentiation state in *Sclerotium rolfsii* concurred with increasing levels of lipid peroxides. This finding prompted the development of a theory supporting that sclerotial metamorphosis is induced by oxidative stress. Growth factors that reduce or increase oxidative stress are expected to inhibit or promote sclerotium metamorphosis, respectively. This theory has been verified by a series of published data on the effect of certain hydroxyl radical scavengers on sclerotial metamorphosis, on the identification and quantification of certain endogenous antioxidants (such as ascorbic acid, β-carotene) in relation to the fungal undifferentiated and differentiated states, and on their inhibiting effect on sclerotial metamorphosis as growth nutrients. In 2004–2005, we developed assays for the measurement of certain redox markers of oxidative stress, such as the thiol redox state, the small-sized fragmented DNA, and the superoxide radical. These new advances allowed us to initiate studies on the exact role of glutathione, hydrogen peroxide, and superoxide radical on sclerotial metamorphosis. The emerging data, combined with similar data from other better-studied fungi, allowed us to make some preliminary postulations on the ROS-dependent biochemical signal transduction pathways in sclerotiogenic filamentous fungi.

Fungal metamorphosis

Competence, induction, and change seem to be the key processes that underline the metamorphic state of fungi (Moore 1998). Competence may be genetic but it is mainly a physiological condition. Induction is the process by which the competent tissue is exposed to conditions that overcome halts to progress, and allow the advance of the next step. Change occurs when the competent tissue is induced, followed by a change in hyphal behavior and physiology. Each metamorphic step brings the fungal tissue to a higher order of differentiation. Differentiated hyphal cells need being in their normal environment, and when removed from it they revert to the mode that characterizes vegetative hyphae. Hyphal differentiation is an unbalanced process compared with the vegetative state. In most hyphal differentiation pathways the balance is tipped to the direction of differentiation by a local microenvironment presumably defined by the local population of hyphae. Another common characteristic is that fungal metamorphosis is expressed as distinct processes recognized at the level of organs, tissues, cells, and cellular components. They are genetically and physiologically distinct and may run in parallel or in sequence. The flexibility in expression of the metamorphic processes allows the fruiting body to react to adverse conditions and still produce differentiation forms (for example spores, sclerotia, conidia) adapted to these conditions. The ultimate flexibility is that the differentiation process can be abandoned in favor of vegetative hyphal growth. A lesser level of flexibility may be in the case where a particular function is carried out by an incompletely adapted cell line.

The molecular mechanisms that control fungal metamorphosis are homologous and analogous to the mechanisms known for animals and plants. A variety of physiological signals and stresses may cause translation level controls to direct competent fungal tissues to undertake specific differentiation processes. Translational trigger could immediately lead to translation of components of highly specific transcription activators and inhibitors, which then regulate gene sequences required for the initiated differentiation. These or their eventual products may be involved in

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feedback fixation of the differentiation pathway. Feedback fixation is the outcome of feedback activation and autoregulation, which together reinforce expression of the whole regulatory pathway to make it independent of the external environmental cues that initiated it. The epigenetic aspects of the network governing fungal metamorphosis start with the feedback fixation, but also include signals from outside the cell.

The fungal extracellular matrix is extensive and complex. Its reaction/interaction with the environment can be communicated to the intracellular environment to modify cytoplasmic activity. Since neighboring cells are components of the external environment, it is expected that the activity of one hyphal cell will be modulated by changes made to the extracellular matrix by a neighboring hyphal cell. Thus, continued progress in differentiation for most fungal cells requires continued reinforcement from their local microenvironment. This may involve production of location-specific and/or time-specific extracellular matrix molecules or hormones and growth factors. Smaller molecules might exert their effects by being taken up into the cell, but uptake is not necessary. Any of theses molecules may also affect relationships between integrins and the existing extracellular matrix. As a result, there could be direct effects on the cytoskeleton, which are able to cause immediate metabolic changes in one or more cellular compartments, or directly influence gene transcription. Connections to the extracellular matrix may also be involved in the control of hyphal branching. By varying extracellular matrix/membrane and/or wall/membrane connections, external signals may be able to specify branch initiation sites. Similarly, internal cytoskeleton architecture could also arrange specific membrane/wall connections to become branch initiation sites. Branch initiation sites specified in these ways may then become gathering sites for the molecules that create a new hyphal tip. The branch would consequently emerge in a position precisely defined by the stimulation of generalized cytoskeleton/membrane/wall connections by positional stimulus. The focus of these hypothetical regulatory activities is the hyphal wall, its surface and the immediate extracellular environment. The key to fungal metamorphosis lies in understanding how that which is outside a hypha can influence that which goes on inside the hypha in a time/place–dependent manner. An important insight to these questions provides the theory of oxidative stress-induced sclerotial differentiation of filamentous fungi (Georgiou 1997), developed within the context of the hyperoxidant state-based general theory of microbial differentiation (Hansberg and Aguirre 1990; Aguirre and others 2005). Both of them are discussed in the present review in the light of the most recent evidence.

Differentiation in microbial eukaryotes is induced by oxidative stress

Cell differentiation in microorganisms manifests itself in a great variety of forms and physiologies. It is related to different growth strategies, various forms of resistance to adverse environmental conditions, different ways of reproduction and differentiation. The general theory of microbial eukaryotic cell differentiation postulates that this phenomenon is triggered by a hyperoxidant state (oxidative stress state), which induces the cell to isolate itself from molecular oxygen (Fig. 1A) (Hansberg and Aguirre 1990; Aguirre and others 2005). The claim that a hyperoxidant state triggers differentiation is a plausible theory, since the great microbial diversity seems to follow the same general principles of controlling or avoiding it. A hyperoxidant state is an unstable pro-oxidant (oxidative) state in which the amount of oxygen-free radicals inside the cell exceeds the cell's capacity to neutralize them. Although not all of them are radicals, the term "oxygen-free radicals" has been used to refer to all species of oxygen (reactive oxygen species, ROS) that are more reactive than O_2 in its ground state or triplet state ($^3O_2$). These are dioxygen in its excited state singlet forms ($^1O_2$) and the partially reduced forms of oxygen, that is superoxide radical ion and its protonated form ($O_2^{\cdot-}$ and $HO_2^{\cdot}$, respectively), hydroxyl radical ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2) (Fig. 2).

Undifferentiation (growth) and differentiation states are stable while the intermediate unstable state is the hyperoxidant state. A cell in a stable state maintains low intracellular O_2 concentration below the level that can trigger the generation of a hyperoxidant state. Consumption of O_2 is maintained and regulated by the availability of reduced electron carrier molecules in a way that ensures stability of either the growth or the differentiation states, although transient fluctuation of intracellular O_2 concentration may occur due to lags in the metabolic responses to sudden changes in the external/internal conditions. Growth occurs only in a stable state. When entrance of O_2 to the cell exceeds its capacity to reduce it, the ROS concentration will increase as well, and a hyperoxidant state will be generated. The instability of this state arises because the antioxidant mechanisms that neutralize ROS are inhibited/inactivated by them. This state can be compensated by lowering the intracellular O_2 concentration either by an increase in its reduction or by a limitation of its entrance into the cell. The pathway the cell will

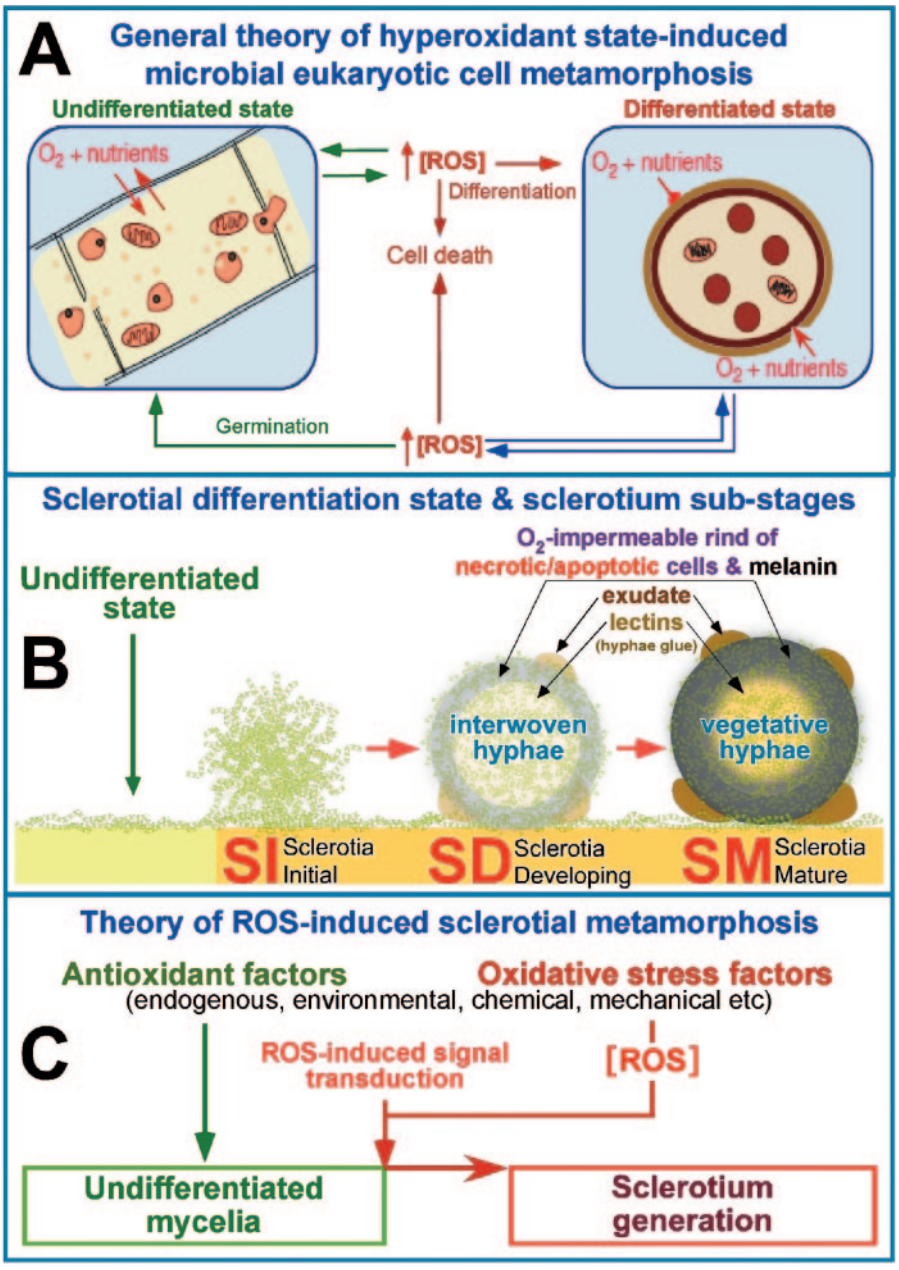


Fig. 1 (**A**) Model for the regulation of microbial eukaryotic cell differentiation by ROS. A hyperoxidant state, a transient and unstable state in which generation of ROS surpasses the antioxidant capacity of the cell, regulates the transition between undifferentiated and differentiated states. A hyperoxidant state has three possible outcomes: (1) the cell compensates with a source of reducing power (nutrients) and returns to the previous stable state, thereby adapting to a more oxidizing condition; (2) the cell differentiates by insulating itself from environmental oxygen; and (3) when adaptation or cell differentiation cannot take place, the reduced internal medium equilibrates with the oxidizing external medium and the cell dies. Cell death enables other cells to either adapt or differentiate. The transition from a differentiated state (such as a spore) to the undifferentiated state (germination) occurs when reducing power derived from nutrient utilization compensates for the generated hyperoxidant state. ROS levels are maintained by a balance between the rate of ROS generation (by mitochondria, NADPH oxidases and other enzymes) and the rate of ROS scavenging (by antioxidant enzymes and by endogenous/exogenous antioxidant molecules). The drawing is adopted from Aguirre and colleagues (2005). (**B**) Differentiated substates (SI, SD, SM) of a typical spherical sclerotium in filamentous fungi (for example *S. rolfsii*), which is a differentiated structure possibly insulating itself from environmental oxygen. When environmental conditions become unfavorable for growth, sclerotia become dormant or inactive and at the SM state they can retain their viability, sometimes for several years, as they possess features that enable them to endure extremes of temperature, desiccation, starvation, harmful irradiation, and biological degradation. (**C**) General principles of the theory of oxidative stress-induced sclerotial metamorphosis in filamentous fungi; it is in compliance with the general theory of hyperoxidant state-induced microbial eukaryotic cell metamorphosis.

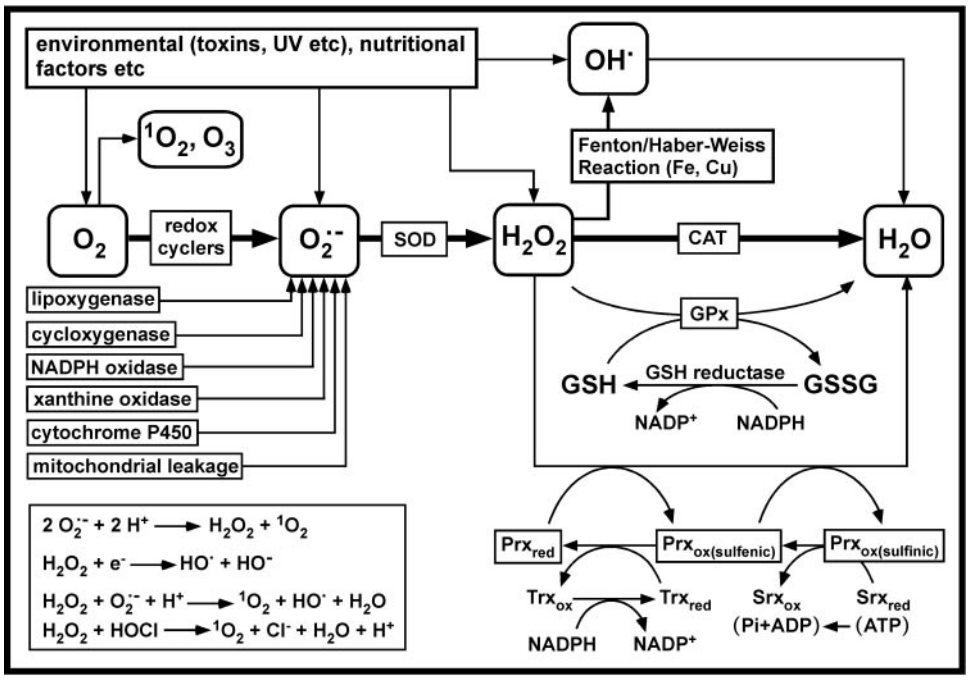


Fig. 2 Mechanisms of ROS production and enzymatic disposal of superoxide and hydrogen peroxide: ROS are produced either by excitation (O_3, $^1O_2$) or by reduction ($O_2^{\cdot-}$, $HO^{\cdot}$, H_2O_2). Species in red are most reactive. Superoxide can be generated enzymically (or not), and can react with it and other radicals, while H_2O_2 reacts with iron sulfur centers and cysteines of certain proteins. However, both superoxide and hydrogen peroxide can spontaneously form singlet oxygen and hydroxyl radicals, which are much more reactive. The main reactions for $^1O_2$ and $HO^{\cdot}$ are shown. Superoxide is dismutated by superoxide dismutases (SODs), and H_2O_2 is decomposed by catalases (CAT), peroxidases (such as glutathione peroxidase and GPx), and by peroxiredoxins (Prx). The thiol of a sensitive cysteine in Prx is oxidized to a Cys-sulfenic acid (Prx_{ox}) and is reduced by reduced thioredoxin (Trx_{red}). The Cys-sulfenic acid in Prx_{ox} can be further oxidized by H_2O_2 to Cys-sulfinic acid, which is reduced back to Cys-sulfenic acid by the reduced sulfiredoxin (Srx_{red}) and ATP.

follow depends on the availability of reducing power (electron donor molecules). Their availability will reduce O_2 intracellular concentration and cause cell reversal to the original state; otherwise, the cell will limit O_2 entrance and reach a different stable state. Starting from growth state it will reach a differentiated state and vice versa. If the cell cannot compensate the hyperoxidant state, by increasing O_2 reduction or limiting its entrance, it will die.

Isolation from external O_2 implies isolation from external water as well as from substances dissolved in it, bringing about a dependence of the cell on endogenous sources for generation of reducing power. When these internal sources become limiting, a hyperoxidant state is generated leading to further isolation from external O_2. Directionality in cell differentiation arises from an increased isolation from the external aqueous medium and from limitations in the availability of the sources of reducing power. A cell differentiation process can go through several differentiated states until it reaches a terminal differentiated state. Such state is characterized by a restricted entrance of O_2 into the cell and by sufficiency of reducing power for maintaining the stable state by reduction to water of the low amount of O_2 entering the cell. There are cellular differentiation processes that can produce two distinct differentiated states, starting from the same stable state. In such processes, the intensity of the hyperoxidant state will lead to a differentiated state that is consequently more isolated from the external medium; a less severe hyperoxidant state leads to a less isolated differentiation state. Cellular mechanisms that limit entrance of O_2 alter irreversibly cell form and surface, making impossible the return of the differentiated state to the previous stable state due to the directionality of the cell differentiation process as well. Starting from any differentiated state, the cell can return to the growth (undifferentiated) state when the isolation from external O_2 is terminated and the cell is in a medium where it can generate sufficient reducing power to reduce the entering O_2 and maintain a stable state.

A differentiated state is a physiological condition in a cell that maintains the cell's stability by isolating it

from environmental O_2. The terminal differentiated state is that which is the most isolated from environmental O_2 under natural conditions. Some intermediate differentiated states (for example sclerotia, cell aggregates, and spherules) are very stable states but are not terminal differentiated states because they can germinate to form more O_2-isolated differentiated states. Differentiated states are stable and thus they may exhibit growth. Due to their isolation, however, they can produce only a limited amount of energy, which is partly used to maintain their isolation from external O_2. Thus, in the more isolated differentiated states growth is restricted or impossible. Cell death or autolysis in a part of a cellular system or cell aggregate constitutes part of the cell differentiation process since it can provide substrates for growth or differentiation of other cells or parts of a cellular system.

Hyperoxidant states have such drastic consequences on cells, that mechanisms preventing these states evolved early in evolution. Prevention of falling into a hyperoxidant state required that cells respond to the danger. Since during a hyperoxidant state many oxidized compounds are formed and/or excreted or liberated from dead cells, some of these substances could function as signals (hormones, signal transducers). Prevention can take the form of triggering antioxidant mechanisms comprising the production of antioxidant molecules (for example, glutathione, vitamin E, ascorbic acid, β-carotene) and enzymes (for example, superoxide dismutase [SOD], catalase, glutathione peroxidase) leading to neutralization of the accumulated ROS (Fig. 2). In addition, hyperoxidant state-driven prevention by cell aggregation or fusion could explain the biogenesis of sclerotia, rhizomorphs, plasmodia, pseudoplasmodia, sex organs, and the aggregation of myxobacteria. Another way of preventing falling into a hyperoxidant state is movement to a less oxidized environment, which could explain why some microorganisms develop flagella before mating or establishing a new colony.

Sclerotial metamorphosis in filamentous fungi and oxidative stress

Sclerotium-forming filamentous fungi are of great agricultural interest because many of them are very important plant pathogens. They are also of great biological interest because they can be viewed as representatives of simple forms of metamorphosis, making them useful models for studying this important biological phenomenon. These fungi are found in the *Ascomycota* and *Basidiomycota*, and they differentiate by asexually producing sclerotia that survive long periods of unfavorable environmental conditions due to their high resistance to chemical and biological degradation (Kuo and Alexander 1967; Coley-Smith and Cooke 1971; Willetts 1971). Sclerotia are asexual, multicellular, firm resting structures. In myxomycetes, the sclerotium is a cluster of enwalled subunits named spherules. Sclerotia of filamentous fungi develop from repeated localized vegetative hyphal branching followed by adhesion of the branches. Outer hyphae form a melanized rind while the inner tissue develop into a cortex of thick-walled cells and a central medulla. Fungal sclerotia are often irregular in shape, but the constituent cells tend to be more or less equal in diameter, in contrast with the myxomycete plasmodia or elongated hyphal cells (Chet and others 1967, 1969; Willetts 1971, 1978, 1997; Chet and Henis 1975; Le Tourneau 1979; Willetts and Wong 1980). Sclerotia of filamentous fungi, in particular, are usually compact bodies of aggregated hyphae, and the degree of their inter-tangling and the presence of a hard, melanized rind and organization of the internal hyphal tissue into different layers distinguishes four sclerotial types: terminal, loose, lateral-chained, and lateral-simple, represented mainly but not solely by *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Sclerotinia minor*, respectively. Specifically, the studied *R. solani* species produces specialized hyphae composed of compact cells, called monilioid, which fuse together to produce hard structures called sclerotia.

Three substates in sclerotia biogenesis were distinguished by ll distinct initials formed from highly proliferating interwoven hyphae, (2) development (SD state), an increase in size, (3) maturation (SM state), characterized by surface delimitation, internal consolidation, melanin pigmentation, and often associated with droplet excretion (exudate) (Fig. 1B). Specifically, the initial sclerotia (SI) in *S. rolfsi* are formed from a single hyphal strand or where two strands cross (Chet and Henis 1975). A spherical shape is formed, even though it consists merely of a network of hyphae that become increasingly more septate (that is, a fungal cell wall cuts across the hypha, dividing it into cells); no hypha coalescence occurs at this state. Differentiated states SD and SM concur by hyphal strand interweaving and sticking together after contact, possibly by excreted lectins (Kellens and Peumans 1991; Kellens and others 1992). At the maturation state, lectins interact with the cell wall-associated putative endogenous receptor leading to the aggregation of mycelium to form sclerotial bodies (Swamy and others 2004). This hyphal coalescence may be facilitated by excretion of exudate (where lectins can be excreted), which is a common phenomenon during the SD and SM states (mainly) of sclerotium biogenesis. Exudate contains

proteins, fatty acids, ammonia, and various enzymes such as melanin-forming polyphenoloxidases and the antioxidant enzymes such as catalase and peroxidase. Its biochemical constitution suggests a cytoplasmic origin and could explain the coating of SM sclerotia by a melanin-pigmented rind, containing necrotic cells as well.

In spite of the biological, ecological, and agricultural significance of the sclerotiogenic filamentous fungi, little is known about the biochemical mechanisms underlying the biogenesis of their sclerotia. Most of the past research on the biogenesis of sclerotia has been conducted on the basidiomycete *S. rolfsii*, and some on the ascomycete *S. sclerotiorum*. At present, it is generally accepted that both nutritional and non-nutritional factors are involved in sclerotial biogenesis (Townsend 1957; Henis and others 1965; Wheeler and Sharan 1965; Griffin and Nair 1968; Trevethick and Cooke 1973; Miller and Limberta 1977). Specific metabolite(s) and morphogenic factors have also been suggested (Wheeler and Sharan 1965; Goujon 1968; Geiger and Goujon 1970). Several hypotheses that have been advanced for the sclerotiogenic filamentous fungi are as follows: that sclerotium biogenesis depends on the accumulation of some (unspecified) metabolite within the hypha (Wheeler and Sharan 1965), that the formation of a protein-like, morphogenic factor in the mycelium induces sclerotia (Goujon 1968, 1969, 1970; Geiger and Goujon 1970) and that sclerotium biogenesis follows the inactivation of an unspecified protein containing −SH groups and Cu^{2+}, which itself represses this process (Chet and Henis 1968).

None of these hypotheses is in agreement with all the experimental observations on the factors affecting sclerotial biogenesis, that have been gathered over the past 50 years. These data when viewed within the perspective of oxidative stress provide a strong indication that the biogenesis of sclerotia is a metamorphic phenomenon that can be explained by the theories of (1) oxidative stress-induced sclerotial differentiation of filamentous fungi (Georgiou 1997) and (2) hyperoxidant state-based microbial differentiation (Hansberg and Aguirre 1990; Aguirre and others 2005). Moreover, the phenotypic states (initiation, development, and maturation) that characterize the sclerotial differentiation of filamentous fungi are in accordance with the main principle of the latter theory, which postulates that the unstable hyperoxidant state induces the microbial cell to isolate itself from molecular oxygen by forming structures, such as sclerotia, which are impermeable to oxygen (Hansberg and Aguirre 1990). It is expected that (1) the more melanized, hard and thick is the external layer of the sclerotium and (2) the less is the ratio of its outer surface over its volume, the less permeable it would be to oxygen (Hansberg and Aguirre 1990).

Factors affecting biogenesis of sclerotia and their relation to oxidative stress

Factors inhibiting sclerotial biogenesis

It has been previously reported that low concentrations of sulfur-containing compounds, such as the amino acids L-cysteine, L-cystine, and L-homocysteine as well as glutathione, inhibit sclerotial biogenesis in *S. rolfsii* (Chet and others 1966; Trevethick and Cooke 1971; Christias 1975). However, these compounds—with the exception of L-cystine, which in the cell can be reduced to L-cysteine—are known to act as free radical scavengers and decrease oxidative stress. Furthermore, glutathione is a substrate of the important antioxidant enzyme glutathione peroxidase, which detoxifies the lipid hydroperoxides in the organisms (Halliwell and Gutteridge 1999). In addition, sclerotia-inhibiting sulfhydryl compounds, such as thioglycolic acid and 2-mercaptoethanol (Christias 1975), are potent reducing molecules, antioxidants and membrane stabilizers (Christophersen 1968; Bartoli and others 1983; Halliwell and Gutteridge 1999).

Other sclerotia-inhibiting substances such as dimethyl sulfoxide, mannitol, glucose, ethanol, and phenylthiourea (Wheeler and Sharan 1965; Chet and Henis 1968; Griffin and Nair 1968; Vega and others 1970; Melhuish and Bean 1971; Okon and others 1972; Wheeler 1972; Christias 1975; Le Tourneau 1976; Hadar and others 1983) are known to function as hydroxyl radical scavengers (Halliwell and Gutteridge 1999). The inhibiting effect of glucose on sclerotial biogenesis is manifested as a delay of sclerotial initiation and maturation with increasing glucose concentrations ranging from 0.1 to 10% (w/v). With glucose >2% (w/v) the number of sclerotia per weight of mycelia decreases. Sclerotia develop only when glucose is depleted from the growth medium (Henis and others 1965; Wheeler and Sharan 1965).

Inhibitors of protein and RNA synthesis, like cycloheximide and 6-methylpurine, when applied to colony margins at low concentrations, have an inhibitory effect on sclerotial biogenesis (Okon and others 1972; Igwegbe and others 1977). Their effect may be exerted on the initiation state of sclerotia, which involves the formation of small discrete sclerotial primordia on the mycelium. The primordia rapidly develop into a white compact hyphal mass made of very thin rapidly growing aerial hyphae with repeated dichotomous branching and fusion of the branches (Townsend and Willetts 1954). Protein inhibitors

can block this rapid development and, therefore, inhibit sclerotial biogenesis. The observed reversal of the 6-methylpurine effect by adenosine, resulting in sclerotial biogenesis (Igwegbe and others 1977), can be explained as being a cause of oxidative stress generated by the formation of $O_2^{\cdot-}$-free radicals during the metabolism of purines via xanthine oxidase (Hassan and Fridovich 1984; Cross and Jones 1991).

Factors promoting sclerotial biogenesis

The induction of sclerotial biogenesis by iodoacetic acid and potassium iodate (Chet and Henis 1968) can be partially explained as being a respective result of the alkylating and oxidizing effect these substances exert on the endogenous antioxidants glutathione and ascorbic acid, and on metabolism in general. Furthermore, iodoacetic acid could alkylate the –SH groups in the active center of glutathione reductase, the antioxidant enzyme that converts the oxidized glutathione to its reduced form (Halliwell and Gutteridge 1999). It could also alkylate the –SH groups in the active center of fructose diphosphate aldolase, a key enzyme in glycolysis (Lehninger 1975). This would result in a decline in the reducing power of the cell and a decrease in the cell's capacity to reduce molecular oxygen to water by the respiratory chain and thereby an increase in oxidative stress. Furthermore, iodoacetic acid might inhibit metallothioneins possibly present in *S. rolfsii*. Metallothioneins in yeast are cysteine-rich proteins that functionally mimic copper–zinc SOD, another important antioxidant enzyme. It has been postulated that such proteins may play a direct role in cellular defense against oxidative stress by functioning as antioxidants (Tamai and others 1993). Therefore, iodoacetic acid could inhibit them by alkylating their cysteine residues.

EDTA and Fe, which promote sclerotial biogenesis in *S. rolfsii* (Chet and Henis 1968), are known to generate ROS when present in chelated form. On the other hand, iron, alone, accelerates lipid peroxidation (Halliwell and Gutteridge 1999). Iron complexed with EDTA catalyzes the formation of $HO^{\cdot}$ radicals via the Fenton reaction and its catalytic effect is ~5-fold higher compared with free iron (McCord and Day 1978; Baker and Gebicki 1984). The induction of sclerotial biogenesis by environmental factors such as light (Trevethick and Cooke 1973; Miller and Limberta 1977), temperature (Hawker 1957; Rudolph 1962), pH (Chowdhury 1946; Townsend 1957; Rudolph 1962; Rai and Agnihotri 1971), oxygen (Griffin and Nair 1968), and by mechanical factors such as injury of the mycelium (Henis and others 1965) can also be explained as being a result of generated oxidative stress.

Light can reduce flavins and flavinoproteins that can react with molecular oxygen to produce $O_2^{\cdot-}$ radicals (Massey and others 1978; Baker and Gebicki 1984). Light is an important generator of oxidative stress via triggering photosensitization reactions between free or protein bound flavins and molecular oxygen, which result in the formation of ROS like hydrogen peroxide, singlet oxygen, superoxide radicals, and hydroxyl radicals (Martin and Burch 1990). Light, besides promoting sclerotial biogenesis in *S. rolfsii*, causes melanin accumulation in sclerotia via induction of tyrosinase (Miller and Limberta 1977), one of the main enzymes in the biosynthetic pathway of melanins (Bell and Wheeler 1986). Since mature sclerotia contain melanin and are also formed in dark in lesser numbers, and since melanins are known to act as free radical scavengers (Halliwell and Gutteridge 1999), their presence in sclerotia of filamentous fungi provides an indirect indication that these morphogenic structures are differentiation products induced by oxidative stress. The mechanism of light-induced sclerotial biogenesis might also involve light-inactivation of antioxidant enzymes as well as of some important enzymes that are involved in cellular oxidative and energy metabolism. Catalase (an important antioxidant enzyme) and lysosomal and mitochondrial enzymes have been known to be inactivated by light (Aronoff 1965; Mitchell and Anderson 1965; Aggarwal and others 1978; Cheng and Packer 1979; Feierabend and Engel 1986). The selenium containing antioxidant enzyme glutathione peroxidase can be inactivated by $O_2^{\cdot-}$, which is an indirect product of light-mediated reactions producing oxygen radicals (Baker and Gebicki 1984).

Sclerotium promotion in *S. rolfsii* by high temperature (Hawker 1957; Rudolph 1962) can be a result of oxidative stress due to denaturation of enzymes related to metabolism of molecular oxygen (Michea-Hamzehpour and others 1980) and to homolytic breakage of some cellular compounds (Baker and Gebicki 1984). Nutrient limitation, another factor promoting sclerotial biogenesis, would cause a fall in the reducing power of the cell, a decrease in the reduction of molecular oxygen in the respiratory chain and a subsequent increase in its intracellular concentration. Oxidative stress and lipid peroxidation would be the result of this phenomenon (Kaschnitz and Hatefi 1975), as well as exposure of the cell to high oxygen concentrations. These factors promote sclerotial biogenesis in *S. rolfsii* (Townsend 1957; Griffin and Nair 1968). Cold could be also a sclerotia-promoting factor since solubility for oxygen at low temperatures increases in water. The same phenomenon is expected to occur in the growth medium and in the cytoplasm

as well, which might cause an increase in oxidative stress and a transition to a hyperoxidant state followed by a sclerotial differentiation state.

The pH of the growth medium has been shown to be very important for sclerotial biogenesis in *S. rolfsii*. The production of sclerotia in this organism and in other sclerotia-producing fungi is favored by acidic pH varying from 4 to 5.5 (Chowdhury 1946; Townsend 1957; Rudolph 1962; Rai and Agnihotri 1971). Furthermore, it is known that acidic growth media inhibit the ATPase proton pump in the plasma membranes of fungi, lower cytoplasmic ATP levels and cause intracellular acidification (Serrano 1984). The pH of the exudate from mature sclerotia of *S. rolfsii* is 4.5 and that of another well-studied fungus *S. sclerotiorum* is 5.4 (Colotelo and others 1971). Since the exudate is rich in nutrients such as carbohydrates, amino acids, proteins, and lipids, it can be assumed to be of cytoplasmic origin, resulting from possible cell death and rupturing of cytoplasmic membranes in the developing sclerotia. Such necrotic cells and remnants of cell walls have been observed in the outer layer (skin) of mature sclerotia in *S. rolfsii* (Chet and others 1969). The possible cytoplasmic origin of the exudate in sclerotia-producing fungi is supported also from the observation that its pH is not far from that of the sclerotial cells from which it originates. For example, the pH of the exudate of *S. sclerotiorum* is 5.4 and that of an aqueous extract of its sclerotia is 6.3 (Colotelo and others 1971), also acidic. From the pH of the exudate and from its assumed cytoplasmic origin, it could be speculated that acidic pH favors possible processes (that is, free radical generation, lipid and protein peroxidation, differentiation) that trigger sclerotial biogenesis in *S. rolfsii*. One such process could be the conversion of the $O_2^{\cdot-}$ to the very active $HO^{\cdot}$ radical, which is maximized at pH 4.8 (Baker and Gebicki 1984; Baker and Gebicki 1986). Furthermore, intracellular changes in pH due to oxidative stress may induce sclerotial biogenesis since they are known to initiate signal transduction; for example, to regulate hormones in plants, to act as second messengers in animal systems and, in general, to be associated with metamorphosis through control of the cell cycle, division, and growth (Kurkdjian and Guern 1989).

It has been proposed that the extracellular pH decrease observed in *S. rolfsii* and *S. sclerotiorum* cultures is due to the excretion of organic acids (mainly oxalic acid) (Maxwell and Bateman 1968b; Vega and others 1970). In *S. rolfsii* in particular, oxalic acid concentration in the growth medium drastically increases during sclerotial metamorphosis, with concomitant decrease of its pH (Maxwell and Bateman 1968a). Precursor of oxalic acid in *S. sclerotiorum* is the endogenous antioxidant erythroascorbate (an analogue of ascorbic acid) (Loewus and others 1995). On the other hand, it has been shown that ascorbic acid decreases during sclerotial metamorphosis of *S. sclerotiorum*, *S. rolfsi*, *R. solani*, and *S. minor* (Georgiou and Petropoulou 2001a, b, 2002; Georgiou and others 2002). Moreover, the increase of secreted oxalic acid concentration in *S. sclerotiorum* is associated with increased concentration of cAMP. In addition, cAMP inhibits sclerotial metamorphosis when externally administered in the growth medium of *S. sclerotiorum* (Rollins and Dickman 1998). It is possible that cAMP acts as a direct/indirect cell proliferation signaling molecule that decreases sclerotial metamorphosis because it causes an extensive, not site specific, cell proliferation all over the fungal mycelial colony.

Mechanical factors, such as injury of the mycelium, are known to promote sclerotial biogenesis at the site of injury in the mycelia of *S. rolfsii* and other fungi (Henis and others 1965; Willetts 1971, 1978). However, injury is expected to expose the injured cells to molecular oxygen, which, in turn, could cause local decrease in the concentration of the endogenous antioxidants (for example, ascorbic acid, glutathione) due to their oxidation. This, then, would cause a local increase in oxidative stress with concomitant triggering of a series of free radical attacks on nucleic acids, nucleotides, thiols, proteins, and lipids (Slater 1984).

Evidence that oxidative stress is involved in sclerotial metamorphosis of filamentous fungi

In 1997, it was shown for the first time that sclerotial biogenesis in *S. rolfsii* was accompanied by the accumulation of high levels of lipid peroxidation products, a well-established oxidative stress indicator. This finding—combined with the realization that the previously reported data on growth factors promoting and inhibiting sclerotial metamorphosis can be chemically categorized as oxidants and reductive agents, respectively—prompted the postulation of a new theory claiming that sclerotial metamorphosis in filamentous fungi is induced by oxidative stress (Fig. 1C) (Georgiou 1997). This theory predicts that growth factors that eliminate or promote oxidative stress are expected to inhibit or promote sclerotial metamorphosis, respectively. It also predicts that endogenous or artificial antioxidants and oxidants that eliminate or promote oxidative stress should inhibit or promote sclerotium metamorphosis, respectively, and that high and low oxidative stress growth conditions should increase and decrease the formation of endogenous antioxidants, respectively.

Table 1 Percent maximum decrease of sclerotial differentiation in the fungal representatives of sclerotial metamorphosis by typical hydroxyl radical scavengers and antioxidants, administered in the growth medium

Fungal strains	*Sclerotium rolfsii*	*Rhizoctonia solani*	*Sclerotinia sclerotiorum*	*Sclerotinia minor*
(A) Hydroxyl radical scavengers salycilate, benzoate, and ethanol (of high, medium, and low scavenging rates) at maximum concentrations (mM, mM, and M, respectively, in growth medium as stated in parentheses)				
Salycilate (high, 1.3×10^{10} $M^{-1}s^{-1}$)	60% (2.5)	63% (3)	48% (3)	29% (1)
Benzoate (medium, 4.3×10^{9} $M^{-1}s^{-1}$)	100% (20)	90% (20)	99% (20)	50% (10)
Ethanol (low, 2.7×10^{9} $M^{-1}s^{-1}$)	90% (1)	60% (0.5)	94% (0.5)	40% (0.5)
(B) Endogenous antioxidants ascorbic acid and β-carotene at maximum concentrations (mM and μM, respectively, in growth medium as stated in parentheses)				
Ascorbic acid	87% (15)	100% (10)	98% (6)	100% (35)
β-Carotene	33% (6)	not determined	23% (10)	33% (6)

0% decrease of sclerotial differentiation is defined as the number and dry weight of sclerotia per fungal colony (for *S. rolfsii*, *S. sclerotiorum*, *S. minor*, and for *R. solani*, respectively) in the absence of the tested antioxidants.

These assumptions have been verified by a series of published and unpublished data over the past 10 years that implicate the involvement of oxidative stress and ROS components in sclerotial metamorphosis of filamentous fungi. Comparisons were made between the vegetative, undifferentiated state of the fungal colony and its differentiated state (comprising the substates SI, SD, and SM). The effect of certain hydroxyl radical scavengers (*p*-nitrosodimethylaniline/salicylate, dimethyl sulfoxide/benzoate/thiourea, and ethanol/mannitol of high, medium, and low scavenging strength, respectively) was studied on sclerotial differentiation and growth of *S. rolfsii*, *S. minor*, *S. sclerotiorum,* and *R. solani*. As predicted by the theory, at low growth non-inhibiting scavenger concentrations there was a correlation of scavenger dose and scavenging strength with increased inhibition of sclerotial differentiation [Table 1(A) shows the effect of selected hydroxyl radical scavengers of high, medium, and low scavenging strength]. Salicylate and *p*-nitrosodimethylaniline (with the highest hydroxyl radical reaction rates) were the most effective inhibitors of sclerotial differentiation and growth (Georgiou and others 2000a, b). Higher scavenger concentrations further inhibited sclerotial differentiation and finally arrested growth, providing antioxidants as antifungal alternatives to traditional fungicides. In these studies, it was proposed that the fungitoxic effect of the antioxidants at high concentrations is an indirect result of their antioxidant mode of action. Antioxidants such as the ones used in this study scavenge hydroxyl radicals (and/or other ROS) by reacting with them and forming intermediate secondary radical products (of lower reactivity than the radical they scavenge) finally leading to stable byproducts that are possibly toxic. Low levels of these secondary radicals (formed at low scavenger concentrations) can be effectively eliminated by the intracellular antioxidant machinery (for example chemical and enzymic antioxidants, high redox pool) of the cell as long as carbon source is available (Halliwell and Gutteridge 1999). At high scavenger concentrations, there may be high accumulation of these secondary radicals (and toxic byproducts), which, under decreased antioxidant intracellular levels due to a carbon-source exhaustion, can act as oxidants, resulting in fungal death.

The indirect establishment of hydroxyl radical involvement in sclerotial metamorphosis raised the question whether hydrogen peroxide is involved as well due to the fact that hydroxyl radical may be produced by hydrogen peroxide via the iron catalyzed Fenton/Haber-Weiss reaction (Halliwell and Gutteridge 1999). In 2000 we developed a method for the *in vivo* measurement of H_2O_2 secretion by filamentous fungi (Georgiou and Sideri 2000) in order to be able to measure H_2O_2 secretion by *S. rolfsii* during sclerotial differentiation (Sideri and Georgiou 2000). Oxidative stress-inducing light and Fe (in the growth medium) had an additive effect on H_2O_2 production levels and on differentiation (sclerotia numbers) of *S. rolfsii*. High H_2O_2 production rates were correlated with high oxidative stress and, most importantly, were observed in less lipid-peroxidized young mycelia and sclerotia (SI and early SD) and not in differentiated and highly lipid-peroxidized old mycelia and sclerotia (SD and SM). Hydrogen peroxide gradients were observed in fungal colonies during their transition from undifferentiated to differentiated state and were not due to changes in glucose concentration or the pH of the growth medium.

The indicators of high oxidative stress (of lipid peroxidation) lipofuscins (Tappel 1975; Halliwell and Gutteridge 1999) were identified in all fungal representatives of sclerotial differentiation types by

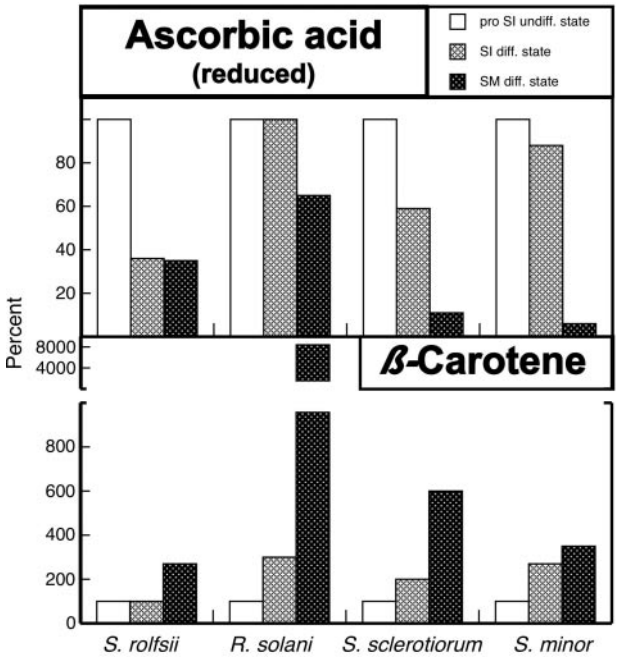


Fig. 3 Percent concentration profiles of the antioxidants ascorbic acid and β-carotene in the fungal representatives of the four types of sclerotial metamorphosis during transition from the undifferentiated to the differentiated state (biogenesis of SI and SM sclerotia).

their excitation and emission spectra (Georgiou and Zees 2001). Lipofuscin pigments in *S. rolfsii*, *R. solani*, *S. minor*, and *S. sclerotiorum* were of similar lipid and protein origin (they showed similar excitation/emission maxima 330/450, 330/450, 330/470, and 330/470 nm, respectively), and their accumulation in the differentiated state of these fungi was 200–600% higher than in the undifferentiated state.

The sclerotium-forming strains of model fungi produce the antioxidants ascorbic acid and β-carotene in levels dependent on oxidative conditions for growth and on metamorphic substates. The percent concentration profiles of the low oxidative stress indicator ascorbic acid (its reduced form) show a decrease in its levels during the transition of the fungi from the undifferentiated to the differentiated state (Fig. 3), which suggests that this transition is followed by an increase in oxidative stress. Exogenously administered ascorbic acid caused a concentration-dependent inhibition of sclerotial biogenesis [Table 1(B)], possibly by decreasing ROS formation in the fungal cells and lowering oxidative stress via its known antioxidant scavenging action (Georgiou and Petropoulou 2001a, b, 2002; Georgiou and others 2002). β-Carotene accumulation in *S. rolfsii*, *S. minor*, and *S. sclerotiorum* is initiated during their transition from the undifferentiated mycelia to the sclerotial differentiation state (Georgiou, Tairis, and others 2001; Georgiou, Zervoudakis, and others 2001; Zervoudakis and others 2003). Data presented in the present review show that this is true also for *R. solani*, the representative fungus of the loose type of sclerotia (Fig. 3). It is proposed that β-carotene is formed and that it possibly reduces the oxidative stress that initiates sclerotial metamorphosis. The antioxidant role of β-carotene in sclerotial metamorphosis is supported by the finding that when it is administered in the growth medium at concentrations that do not inhibit growth, it causes a concentration-dependent reduction of oxidative stress (lipid peroxidation, data not shown) of *S. rolfsii*, *S. minor*, and *S. sclerotiorum* undifferentiated mycelia, and an equally proportional reduction of sclerotial differentiation [Table 1(B)].

In 2004–2005, we developed assays for the measurement of certain markers/components of oxidative stress and ROS. Specifically, we developed assays for the quantification (1) of the various molecular components of the thiol redox state (TRS) (Patsoukis and Georgiou 2004, 2005), (2) of the apoptosis/necrosis related small size fragmentated DNA (Georgiou, Patsoukis, and others 2005) and (3) of the main ROS component superoxide radical (Papapostolou and others 2004; Georgiou, Papapostolou and others 2005; Patsoukis and others 2005). These new advances allowed us to evaluate in more detail the involvement of oxidative stress in the sclerotial metamorphosis of filamentous fungi.

Preliminary data have shown that TRS changes drastically in the sclerotiogenic fungi during their transition from the undifferentiated to the differentiated state. For example, the main TRS component glutathione (GSH) and the total reduced thiol proteins (PSH)—most antioxidant enzymes require reduced –SH groups for their activity—form decreasing concentration gradients during transition to the differentiated state (Fig. 4). This is a strong indication of a progressively increasing oxidative stress. The metamorphosis-inhibiting antioxidant role of GSH has been shown by exogenous GSH and its precursors cysteine (CSH) and *N*-acetyl cysteine [Table 2(A)]. The direct/indirect antioxidant role of these TRS modulators is under investigation.

The gradual increase of oxidative stress in fungi with sclerotial metamorphosis has been also shown by the increased amount of small size fragmented genomic DNA during their transition to the differentiated state (Fig. 5). This is an important finding in light of the fact that (1) severe DNA damage (especially the non-repairable double strand DNA breakage) can lead to cell necrosis and apoptosis and (2) that necrotic cells have been observed in mature sclerotia

in *S. rolfsii* (Chet and others 1969). In necrosis, a random digestion of DNA occurs, while apoptotic events lead to a controlled mono-nucleosomal and oligo-nucleosomal length fragmentation of DNA (200–1000 bp, as multiples of 180–200 bp) (Halliwell and Gutteridge 1999).

Superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and lipid hydroperoxides (LOOH) are direct indicators of oxidative stress and constitute some of the most important components of ROS. Moreover, it has been proposed that these components have direct/indirect role in signal transduction pathways that affect cell differentiation, proliferation, apoptosis, and cycle, which will be discussed in detail in the next section titled "ROS signaling in fungal metamorphosis: sclerotial metamorphosis". Continuous or converging (at the undifferentiated-differentiated transition point, that is, the pro SI substate) gradients of these ROS components were detected in the tested fungal representatives of sclerotial metamorphosis (Fig. 6). The formation rate of $O_2^{\cdot-}$ is the lowest at the initiation (SI) of sclerotial differentiation (in *S. rolfsii* and *S. sclerotiorum* at SM as well). This finding is in agreement with the highest excretion of H_2O_2 in the growth medium at the SI differentiation state, given the fact that H_2O_2 results mainly from dismutation of $O_2^{\cdot-}$ (Halliwell and Gutteridge 1999). The role of $O_2^{\cdot-}$ in sclerotial metamorphosis has been shown indirectly by the decrease of sclerotial numbers caused by its scavenger Tempol, a mimetic of the antioxidant enzyme SOD [Table 2(B)].

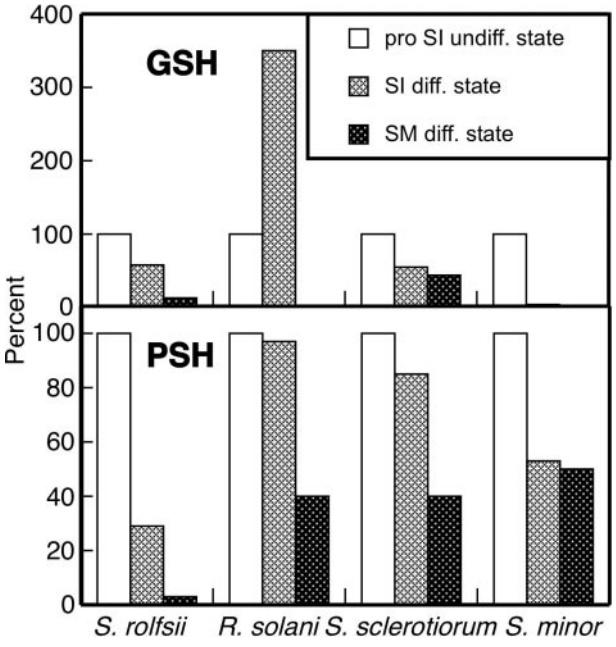


Fig. 4 Percent concentration profiles of the reduced components of thiol redox state (TRS) glutathione (GSH) and of reduced protein thiols (PSH) during transition from the undifferentiated to the differentiated state of the fungal representatives of sclerotial metamorphosis.

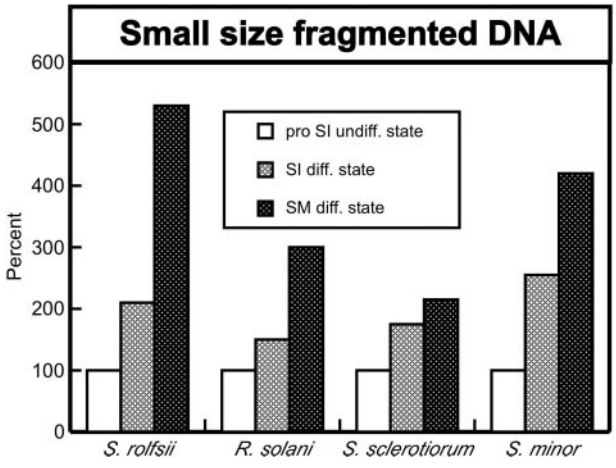


Fig. 5 Percent concentration profiles of small size fragmented DNA formed during transition from the undifferentiated to the differentiated state of the fungal representatives of sclerotial metamorphosis. This is an indication that cell apoptotic/necrotic events took place, possibly resulting in the formation of the internal hyphal core protective and O_2-impermeable rind of the sclerotium.

Table 2 Percent maximum decrease of sclerotial differentiation in the fungal representatives of sclerotial metamorphosis by thiol redox state (TRS) modulators and the superoxide dismutase (SOD)-mimetic Tempol, administered in the growth medium

Fungal strains	*Sclerotium rolfsii*	*Rhizoctonia solani*	*Sclerotinia sclerotiorum*	*Sclerotinia minor*
(A) TRS modulators at maximum concentrations (mM in growth medium, as stated in parentheses)				
Glutathione	100% (5)	100% (5)	100% (20)	52% (20)
N-acetyl cysteine	100% (15)	100% (10)	72% (40)	30% (90)
Cysteine	92% (2)	65% (12)	90% (15)	63% (20)
(B) SOD-mimetic at maximum concentration (0.3 mM in growth medium)				
Tempol	22%	20%	30%	33%

0% decrease of sclerotial differentiation is defined as the number and dry weight of sclerotia per fungal colony (for *S. rolfsii*, *S. sclerotiorum*, *S. minor*, and for *R. solani*, respectively) in the absence of the tested antioxidants.

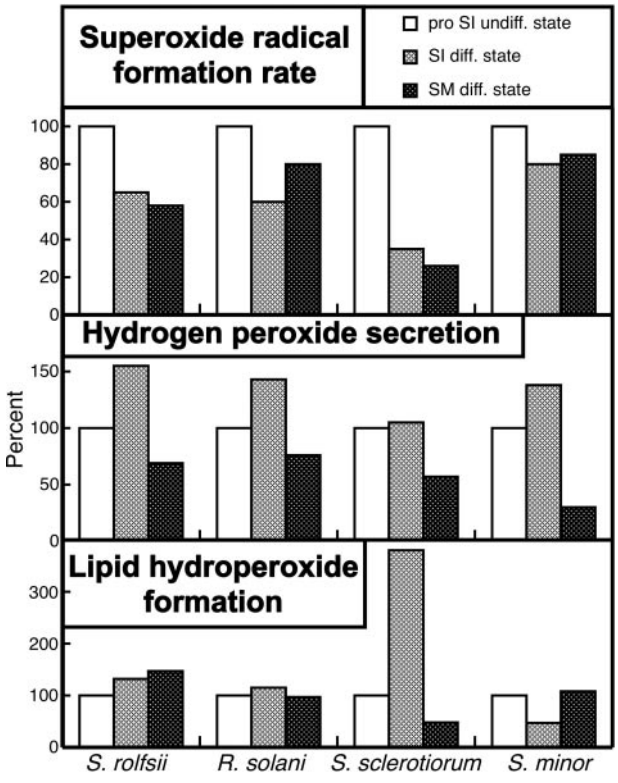


Fig. 6 Percent concentration profiles of the ROS components superoxide radical (rate of production), hydrogen peroxide (secreted in growth medium) and lipid hydroperoxides (formed in hyphae due to ROS action) during transition from the undifferentiated to the differentiated state of the fungal representatives of sclerotial metamorphosis.

As it has been mentioned in the previous section titled "Sclerotial metamorphosis in filamentous fungi and oxidative stress", sclerotia initials (SI) are characterized from highly proliferating interwoven hyphae. This phenomenon may be attributed to a localized production of H_2O_2 in SI sclerotia, as it has been shown for *S. rolfsii* (Sideri and Georgiou 2000). Site-specific cell proliferation may not be restricted only to H_2O_2, since a dramatic increase of lipid hydroperoxides (LOOH) was observed in SI sclerotia of *S. sclerotiorum* (and to a much lesser degree in *S. rolfsii* and *R. solani*). The cell proliferating roles of H_2O_2 and LOOH have been confirmed by unpublished data from our laboratory, which show that *S. sclerotiorum*, *R. solani*, *S. rolfsii,* and *S. minor* form undifferentiated thick mycelial colonies when their growth is enhanced by certain concentrations of H_2O_2 and cumene hydroperoxide (an artificial LOOH analogue).

Although the theory of oxidative stress-induced sclerotial metamorphosis has been tested only in certain phytopathogenic filamentous fungi, it may also apply to other plant pathogenic fungi (for example *Aspergillus*, *Botrytis*, *Claviceps*, *Cylindrocladium*, *Macrophomina*, *Phymatotrichum*, *Typhula,* and *Verticillium*, particularly those that form less differentiated microsclerotia similar to those of *R. solani*), and to sclerotium forming non-phytopathogenic fungi (for example *Bulbillomyces*, *Collybya*, *Coprinus*, *Elaphomyces*, *Morchella*, *Pleurotus*, *Polyporus*, *Wolfporia*).

ROS signaling in fungal metamorphosis: sclerotial metamorphosis

It has been known that ROS influence molecular and biochemical processes and signal transduction pathways, which affect proliferation, differentiation, and death in fungi, and in a variety of other organisms (Allen and Tresini 2000; Schafer and Buettner 2001). These events are closely associated with alterations in the expression of a large number of genes. Although the participation of ROS in intracellular signaling is widely documented, it has been difficult to understand how their involvement meets the requirement of signaling for specificity, given the fact that they can react with almost every biomolecule. Today, it is accepted that ROS specificity in signaling is chemically submolecular and functionally multimolecular (Nathan 2003). In the case of proteins, chemical specificity is illustrated by the preferred reactivity of ROS with Cys-SH, Met-S, Tyr-OH, and protein-[Fe–S] and protein-heme. Because the chemical targets of ROS are basic domains of numerous proteins, multimolecular reactivity is an inescapable consequence (Nathan 2003). ROS-mediated signal transduction pathways in fungi and in higher organisms can be separated into three sequential stages: (1) Receptors mediating/promoting ROS responses, (2) ROS-targeted signal molecules, and (3) ROS-mediated morphogenic processes (Thannickal and Fanburg 2000). In fungi, these processes have been studied mainly in unicellular yeast, in slime molds, and to a lesser degree in certain multicellular fungi such as *Neurospora crassa*. Very little is known about the signaling processes that govern sclerotial metamorphosis in filamentous fungi. It may be assumed that they will not differ in principle from those of other fungi, since these processes are supposed to be conserved (more or less) in these microorganisms.

Receptors mediating/promoting ROS responses

A variety of cytokines, growth factors, nutritional signals, as well as UV light, radiation, and osmotic balance, have been reported as signals capable of causing ROS-stress responses in various microorganisms

(Thannickal and Fanburg 2000). Besides malfunctioning mitochondria, fungal NADPH/NADH oxidases (NOXs) (analogous to the mammalian NOXs) are potential sources of ROS, which are involved in cell proliferation and differentiation, signal transduction and ion transport. Interestingly, within fungi there is a correlation between the presence of *nox* genes and the capability of developing fruiting bodies (multicellular structures) (Finkel 1998; Aguirre and others 2005). Cycloxygenases and lipoxygenases are potential $O_2^{\cdot-}$-generators in some fungi and mainly in plants and animals (Brash 1999; Oliw 2002).

Cytokine receptors

Cytokine receptors fall into a large and heterogeneous group of receptors that lack intrinsic kinase activity and are not directly linked to ion channels or G proteins (Thannickal and Fanburg 2000). Despite a wealth of information on the downstream signal cascades in microorganisms, it became clear that there are distinct types of upstream activators (or receptors) for the transmission of the signals, and distinct intracellular effectors in each pathway. For example, it was shown that yeast strains disrupted in the plasma membrane sensor proteins Wsc1 and Mid2, which are known to be affected by nutrients, low osmolarity, and high temperature, are very sensitive to H_2O_2, suggesting that Wsc1 also plays a role in oxidative stress response (Staleva and others 2004).

Sensor/receptor kinases

A number of growth factors that bind receptor tyrosine kinases (RTKs) and receptor serine/threonine kinases have been shown to generate intracellular ROS essential for mitogenic signaling in mammalian cells (Thannickal and Fanburg 2000). Such oxidative stress-affected receptor kinases also have been found in yeast. For instance, *S. cerevisiae* membrane regulator Sln1 (with histidine kinase activity) is involved in osmotolerance—the high osmolarity growth (Hog) pathway—and also in a signal transduction pathway affected by environmental stresses and oxidative stress. Sln1 also regulates the expression of the antioxidant thiol thioredoxin 2 (Li and others 1998). *Schizosaccharomyces pombe* H_2O_2-sensing pathways contain a plethora of kinases such as the hybrid histidine kinases Mak1, Mak2, Mak3, which activate various downstream MAPKs (Aguirre and others 2005). Moreover, the phosphatidylinositol kinase-related kinases TORs (targets of rapamycin) of *S. pombe* (involved in the coordination between nutritional or mitogenic signals and cell growth) are expressed under stress (starvation, cold, and osmotic and oxidative stress) (Weisman and Choder 2001).

G protein-coupled cell surface receptors

A number of ligands that bind to these receptors have been shown to generate ROS in different cell systems of higher organisms (Thannickal and Fanburg 2000) and microorganisms (Staleva and others 2004). For example, mating in haploid *S. cerevisiae* occurs after activation of the pheromone transmembrane receptors Ste2/Ste3 coupled to a heterotrimeric G protein. It was found that the antioxidant *N*-acetyl-cysteine completely inhibits activation of the pheromone-responsive genes FUS1 and RLM in yeast, suggesting their relation to oxidative stress as indicated as well by the activation of transcription of RLM1 by H_2O_2 (Staleva and others 2004).

ROS-targeted signal molecules

Although a large number of signaling pathways appear to be regulated by ROS, the signaling molecules targeted by ROS are less clear. After the first step of a signaling cascade, which involves the activation of receptors, the signal is transmitted to the nucleus where it activates the transcription factors that regulate gene expression. Throughout this process it has been found that the main targets of oxidative stress are receptor kinases, phosphatases, regulatory proteins, and membrane lipids (Thannickal and Fanburg 2000).

Receptor kinases

The most studied are the (MAP) kinase subfamilies ERK, JNK/SAP kinase, p38 kinases, and BMK/ERK5. It has been found that many MAPK-dependent pathways contain redox sensitive sites (Allen and Tresini 2000). Moreover, non-RTKs belonging to the Src kinases, and Janus kinase (JAK) family are regulated by various ROS. MAP kinases contain redox sensitive sites such as tyrosine residues, which are potential targets of ROS resulting to tyrosyl radicals and dityrosine cross-linking. A conserved family of fungal MAPKs (*Saccharomyces cerevisiae* Hog1, *S. pombe* Spc1, and possibly in filamentous fungi) is activated by many stress signals, including oxidative stress (Aguirre and others 2005). In the yeast *S. pombe*, H_2O_2-sensing pathways contain many downstream receptor kinases such as Wis4, Wis1, and MAPK Spc1-Sty1, which can activate the phosphotransfer protein Mpr1 and the response regulatory proteins such as Prr1. Upon activation of the above H_2O_2-sensing pathways, numerous genes are expressed in order to combat oxidative and other types of stress (Aguirre and others 2005). Homologs of all components of the H_2O_2-sensing pathway described in *S. pombe* are found in *Aspergillus nidulans* and *N. crassa* (Aguirre and others 2005). MAP kinases in *Candida albicans* and *M. grisea*

have been shown to control differentiation (conidiation) (Xu 2000).

Phosphatases

Serine/threonine and tyrosine phosphatases (PTPs). They are important in most signal transduction pathways, because their failure to antagonize kinase actions can disrupt normal cellular functions. Phosphatases possess redox sensitive catalytic domains containing cysteine residues, oxidation or mutation of which result in phosphatase inactivation (for example, H_2O_2 is a potent inhibitor of PTPs) (Allen and Tresini 2000). In yeast, protein phosphatase PP2A is required for nuclear accumulation of the transcription factor Msn2p in response to oxidative stress (Santhanam and others 2004).

Response and transcriptional regulators, and second messengers

The DNA-recognition and binding of the transcription factor NF-kB is redox mediated, and is involved in the regulation of numerous genes including acute phase proteins, cell surface receptors, and cytokines of higher organisms (Burdon 1995; Thannickal and Fanburg 2000). In addition, transcription activator protein-1 (AP-1, a complex of dimerized Fos–Jun or Jun–Jun proteins) is activated by exogenous oxidants and ligand-induced ROS, and by some antioxidants (Thannickal and Fanburg 2000). Similar stress response regulatory molecules have been found also in fungi. For example, in the yeast *S. cerevisiae* the DNA-binding Yap family (of eight members) of b-ZIP proteins has been involved in oxidative stress: Yap1 regulates ROS for example ($O_2^{\bullet-}/H_2O_2$)-induced stress generated by redox cyclers, thiol oxidants, and alkylating agents, and cadmium. Upon direct effect of oxidative stress (H_2O_2), Yap1 enters and remains in the nucleus (due to an intra Cys–Cys bridge formation), where it induces the expression of H_2O_2-scavenging genes (Garreau and others 2000; Hasan and others 2002). In the yeast *S. pombe,* phosphotransfer protein Mpr1 and response regulators Mcs4 and Prr1 are involed in the activation of the transcription factors Atf1 and Pap1, which are responsible for the expression of the H_2O_2-dependent genes (Aguirre and others 2005). Intracellular concentrations of second messengers cAMP and Ca^{2+} are modulated by ROS, the former via ROS-inhibition of adenylate cyclase (Burdon 1995; Gesqiere and others 2000) and the latter via inhibition of Ca^{2+} pumps (Burdon 1995; Thannickal and Fanburg 2000). ROS-modulated calcium has been also observed in *A. nidulans* (Greene and others 2002). In yeast *Kluyveromyces lactis*, the Golgi P-type Ca^{2+}-ATPase, Pmr1p, is the major component for calcium homeostasis and its deactivation leads to a new phenotype that exhibits traits of ongoing oxidative stress (H_2O_2 sensitivity), reduced glycosylation, cell wall defects, and alterations of mitochondrial metabolism (Uccelletti and others 2005). Gene groups responsive to H_2O_2 (and to certain ROS generators), GSH/GSSG, and $O_2^{\bullet-}$ in *A. nidulans* are those coding for "PBS2 like MAPK" homologue, PSK2 kinase homologue, AtfA transcription factor, and many elements of ubiquitin tagging, cell division cycle regulators, translation machinery proteins, defense and stress proteins, transport proteins as well as many enzymes of the primary and secondary metabolisms. In addition, a separate set of genes encoding transport proteins, CpcA and JlbA amino acid starvation-responsive transcription factors, and some elements of sexual development and metamorphic sporulation was ROS responsive (Pocsi and others 2005).

Membrane lipids

It is known that $O_2^{\bullet-}$, $HO^{\bullet}$ and H_2O_2 cause peroxidation of membrane lipids. It has been shown that ROS-mediated cellular signaling can be carried out by lipid hydroperoxides (LOOH) and their decomposition product 4-OH-nonenal (4-HNE) (Yang and others 2003). For example, fungal intracellular signaling mechanisms (for example cell cycle arrest in yeast) are affected by LOOH and 4-HNE (Wonisch and others 1998).

ROS-mediated metamorphic processes

Cell cycle

Recent research has proved that the effects of ROS on fungal proliferation or cell arrest are similar to those exerted on higher organisms (Flattery-O' Brian and Dawes 1998; Schafer and Buettner 2001). For example, it has been shown that yeast can be arrested to G_2-phase in response to ROS (Flattery-O' Brian and Dawes 1998). Moreover, it has been reported that cell arrest-regulating protein kinase YakA in the slime mold *Dictyostelium discoideum* is activated by H_2O_2, which can also trigger cAMP synthesis and activation of another PKA (Taminato and others 2002). Variations of H_2O_2 intracellular concentration can trigger cell proliferation in fungi (Aguirre and others 2005).

Apoptosis

Although in the past apoptosis was believed to be confined to multicellular higher eukaryotes, today, there is increasing evidence that it also exists in ROS-stressed fungi (filamentous and unicellular, for example *Aspergillus fumigatus*, *A. nidulans*, and yeasts). For instance, the antifungal protein PAF from

Penicillium chrysogenum promotes intracellular accumulation of ROS, disintegration of mitochondria, and an apoptosis-like cell death (Leiter and others 2005). Apoptosis can be induced in yeast by depletion of glutathione or by low external doses of H_2O_2. Yeast can also be triggered into apoptosis by a mutation in CDC48 (an ATPase in ER, nuclear membrane, and cytoplasm) (Madeo and others 1999). In the fungal phytopathogen *Colletotrichum trifolii*, the mutationally activated oncogenic fungal Ras (DARas) elevates levels of ROS and causes abnormal fungal growth and metamorphosis and eventual apoptotic-like cell death, inhibited by the osmolytic amino acid proline (shown to scavenge intracellular ROS) (Chen and Dickman 2005).

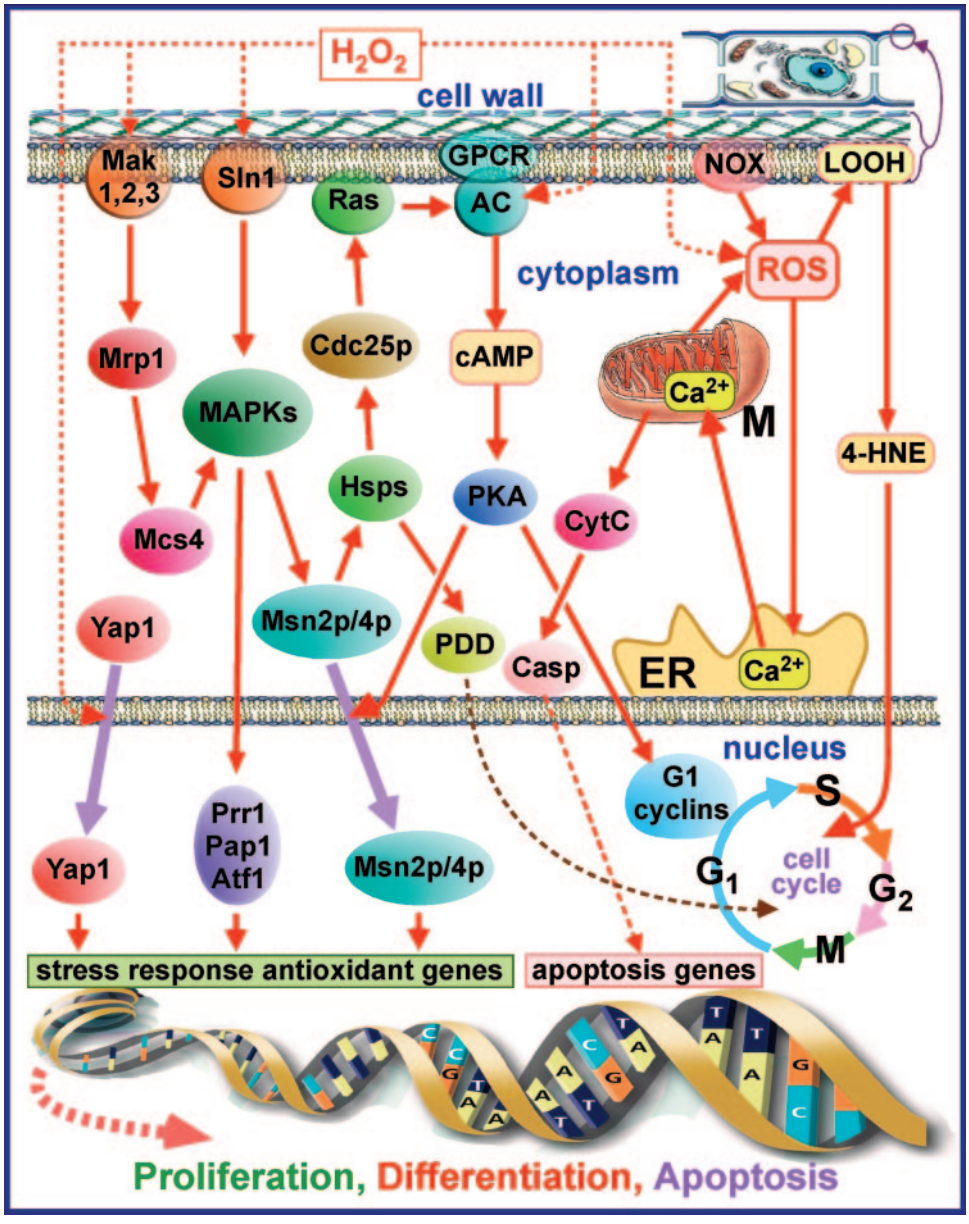
Differentiation (metamorphosis)

It has been proposed that fungal differentiation—and eukaryotic microbial differentiation, in general—may be triggered by the transition from the undifferentiated state to an intermediate unstable hyperoxidant state and finally to a more stable differentiated state. The switch to the hyperoxidant state occurs by a transient increase in ROS levels beyond the cellular capability to neutralize them (Aguirre and others 2005). We believe that this is not an on/off switch but an outcome of the gradual formation of oxidant and antioxidant (enzymic and non-enzymic) concentration gradients (resulting from the gradual exhaustion of extracellular/intracellular electron donors), certain molecular components of which will interact (in a concentration-dependent manner) with molecular components of the metamorphosis-related signal transduction pathways. This is in agreement with the hypothesis that metabolic, redox, and ROS generation gradients can be interrelated and influence transcriptional and translational controls of gene expression (Allen 1991). Our theory is strengthened by the finding that the activity of some transcription factors is highly dependent on the cell redox environment, which is constituted by the sum of the products of the reduction potential and reducing capacity of certain linked redox couples (such as GSSG/2GSH, NAD^+/NADH, and $NADP^+$/NADPH). ROS-induced changes in these redox couples can influence signal transduction, DNA, RNA, and protein synthesis, enzyme activation, and even regulation of the cell cycle (Schafer and Buettner 2001). These changes are also expected to induce the formation of concentration gradients of endogenous antioxidants (such as reduced ascorbate, β-carotene, GSH, and PSH), and of oxidants (such as the lipid peroxidation products LOOH and lipofuscins, $O_2^{\cdot-}$, and H_2O_2). The existence of such gradients in the experimental models of sclerotial metamorphosis in filamentous fungi has been already mentioned (Figs 3–6).

Several studies in various fungi have shown a correlation between differentiation/no-differentiation processes and the upregulation of specific antioxidant enzymes, such as SODs, catalases, bifunctional catalase-peroxidases, and peroxiredoxins. For example, a SOD gene is induced during sporulation in *Colletotrichum graminicola* and spherulation (biogenesis of microsclerotia [Allen 1991]) in the slime mold *Physarum polycephalum* (Aguirre and others 2005). In the latter fungus, MnSOD activity decreases before differentiation, but as differentiation proceeds the activity increases by ~46-fold; in a non-differentiating mutant strain no significant change in MnSOD occurred. Addition of the ROS generator paraquat to the differentiating and non-differentiating strains revealed that SOD activity could be induced in both strains. The increase in SOD activity observed in the differentiating strains could be induced by the increase in the rate of $O_2^{\cdot-}$ generation, while the failure of the non-differentiating strain to significantly alter its SOD activity under identical culture conditions was due to its inability to generate $O_2^{\cdot-}$. Moreover, the activity of another antioxidant enzyme glutathione peroxidase (GPx) in the slime mold *P. polycephalum* is initially very low in the undifferentiated microplasmodial state but it increases slightly during spherulation (Allen and Balin 1989). Similarly several genes of the antioxidant enzyme catalase in the fungi *A. nidulans* (catA–D) and *N. crassa* (cat-1–3) are expressed at different metamorphic states. In summary, (1) the induction of antioxidant enzymes in structures that are undergoing metamorphosis and (2) the association of specific antioxidant enzymes with fully differentiated structures suggest that ROS are produced at the start and during cell differentiation in fungi.

Besides the changes in the antioxidant enzyme activities, other approaches have related the presence of ROS with fungal metamorphosis. In *N. crassa*, increased formation of ROS occurs at the start of each cell-differentiation step, leading to conidial metamorphosis, whereas $^1O_2$ is produced at the start of conidial germination. Water-soluble antioxidants that can enter the cell were shown to inhibit ROS and conidiation in *N. crassa*, and this is consistent with the idea that ROS are needed for the differentiation process to occur. The differentiation of microplasmodia into microsclerotia in the slime mold *P. polycephalum* occurs with an 80% decrease of GSH concentration. Addition of GSH to the culture medium retarded sclerotization in this microorganism (Allen and Balin 1989). Similar observations were made in *S. sclerotiorum*, *R. solani*, *S. rolfsii,* and

S. minor with certain hydroxyl and superoxide radical scavengers, endogenous antioxidants, and thiol modulators (Tables 1 and 2). In line with these observations, it is expected that a lack of antioxidant enzymes would result in higher levels of ROS and cell differentiation. In *N. crassa*, the inactivation of catalase-3 gene results in enhanced protein oxidation, higher carotenoid levels, hyphae adhesion, and higher amounts of aerial hyphae and conidia. The lack of copper-zinc SOD (and expected increase of ROS) in this fungus also increases carotenoid production and alters the polarity of the sexual fruiting bodies. Increased levels of β-carotene under growth conditions of increased oxidative stress were also observed in *S. sclerotiorum*, *S. rolfsii,* and *S. minor* (Georgiou, Tairis, and others 2001; Georgiou, Zervoudakis, and others 2001; Zervoudakis and others 2003), and in *R. solani* (unpublished data). Despite the evidence indicating that ROS are related and probably required for cell differentiation in fungi, the sources of these ROS and their contribution to this process remain obscure. It is clear, however, that mitochondria and NADPH oxidases are important cellular sources of ROS in fungi (Aguirre and others 2005). Lipid peroxidation in fungi has been implicated in a large number of cellular processes including cell proliferation and differentiation. Many fungi exhibit a marked increase in lipid peroxidation during differentiation. For instance, in *P. polycephalum* lipid peroxides increase during the initial phases of spherulation and then decrease as differentiation is completed. On the contrary, in a non-differentiating strain of *P. polycephalum* the lipids do



not peroxidize significantly before the organism dies in the differentiation medium (Allen and Balin 1989). A marked increase in lipid peroxidation has been observed in *S. rolfsii* during its differentiation state (Georgiou 1997).

Depending on the concentration, H_2O_2 responsiveness in *S. pombe* is currently perceived through four different pathways (Nguyen and others 2000; Buck and others 2001; Veal and others 2004; Vivancos and others 2004; Aguirre and others 2005): (1) a multistep phosphorelay involving Mak1 and Mak3 sensor kinases, Mpr1 phosphoprotein and Mcs4 response regulator transmit the H_2O_2 signal to the Spc1-Sty1 MAPK, which phosphorylates the transcription factor Atf1; (2) the 2-Cys peroxiredoxin Tpx1 transmits H_2O_2 signal directly to Spc1-Sty1 (Veal and others 2004); (3) Mak1 sensor kinase has been proposed to relay H_2O_2 stress through Mpr1, the only phosphotransfer protein in *S. pombe*, to response regulator Prr1; (4) Pap1 transcription factor might receive the H_2O_2 oxidation signal through Gpx1 in a mechanism analogous to *S. cerevisiae* Gpx3 and Yap1 (Delaunay and others 2002; Toledano and others 2004).

On the signal transduction level of the ROS-targeted signal molecules, it has been shown that cAMP is implicated in oxidative stress-related fungal differentiation, especially in the sclerotiogenic filamentous fungi. For example, it has been shown that if *S. sclerotiorum* growth takes place in the presence of the antioxidant caffeine, the levels of cAMP in mycelia are increased, while administration of cAMP decreases or eliminates the production of sclerotia (Rollins and Dickman 1998). Preincubation of *R. solani* with cAMP (and ATP) significantly inhibits sclerotiogenesis (Uno and others 1985). Similarly, changes in intracellular cAMP levels affect sclerotiogenesis in *S. rolfsii* (Hadar and others 1983). In *Dictyostelium discoideum* increase of cAMP and subsequent activation of PKA has been found to cause sporulation (Anjard and others 1998). An explanation of how cAMP is related with the inhibition of fungal differentiation may be based on the fact that stress responses in yeast can be mediated by the cAMP/PKA (cAMP-dependent protein kinase) pathway. cAMP is synthesized by adenylate cyclase, which is encoded by the CYR1/CDC35 gene. Increased concentration of intracellular cAMP activates the cAMP-dependent protein kinase (PKA) by binding to its regulatory subunit (encoded by the BCY1 gene) and by dissociating its catalytic subunits (encoded by TPK1, TPK2, and TPK3). The activation of adenylate cyclase can occur through different pathways, involving a G-protein coupled receptor (GPCR)

Fig. 7 ROS-induced signal (cAMP/H_2O_2) transduction pathways possibly involved in the sclerotial metamorphosis of filamentous fungi (a typical filamentous cell with its membrane and its cell wall is shown), as adopted from published yeast ROS-stress responses (described in the text): Oxidative stress response (antioxidant) genes are activated by the transcription factors Msn2p/4p, which, in *S. cerevisiae*, are translocated in the nucleus after being activated via an MAPKs cascade pathway (e.g Hog1) that is previously activated by the H_2O_2-activated sensor receptor kinase Sln1. Alternatively, these genes, in *S. pombe*, may be activated by the transcription factors Pap1, Prr1, and Atf1, which are activated via a different MAPKs cascade pathway (for example Wis4/Win5, Spc1/Sty1). Here, the signal is transmitted to MAPKs via the H_2O_2-activated sensor receptor kinases Mak1, Mak2, Mak3, and the sequentially activated phospotransfer protein Mrp1, and the response regulator Mcs4. Hydrogen peroxide can directly affect transcription factor Yap1 (in *S. cerevisiae*) by oxidizing its thiol groups and thus resulting to its nuclear translocation and activation of the stress response antioxidant (SRA) genes. Gene groups responsive to H_2O_2 and $O_2^{\cdot-}$ in *Aspergillus nidulans* are those coding for "PBS2 like MAPK" homologue, PSK2 kinase homologue, and for AtfA transcription factor (Pocsi and others 2005), all homologue to those found in *S. pombe*.
ROS can also directly inhibit the cAMP-producing adenylate cyclase (in *S. cerevisiae*). Activation of adenylate cyclase can occur through a (Glucose) G protein-coupled receptor (GPCR) system or by the membrane bound GTP-hydrolysing (GTP receptor) Ras1p and Ras2p (Ras). The GPCR activates adenylate cyclase (AC) in response to high glucose levels. Upon adenylate cyclase activation, cAMP levels increase (or by external administration of cAMP) and subsequently activate cAMP-dependent protein kinase (PKA), which inhibits the nuclear translocation of the transcription factors Msn2p/4p, resulting in non-expression of antioxidant enzymes-encoding genes and possible accumulation of $O_2^{\cdot-}$/H_2O_2 (and possible cell proliferation in sclerotiogenic fungi). Msn2p/4p activate the heat shock proteins (Hsps), which, in turn, activate the guanine nucleotide exchange factor for Ras, Cdc25p, Ras (via Cdc25p), and AC (via Ras). Upon oxidative stress conditions, the ROS-induced accumulation of oxidized proteins would recruit Hsps in order to lead them to a proteasome-dependent degradation (PDD). Thus, Hsps will not be available for activation of the cAMP-PKA pathway, and cAMP, which is required for the transcription of G1 cyclins (required for cell proliferation), will not be produced. This will result in G1-phase cell arrest. Cell cycle disruption can be also caused by 4-HNE, the product of degradation of lipid hydroperoxides (LOOH), which can be formed by ROS [generated by NOXs, possible malfunction of mitochondrial respiratory chain etc (Fig. 2)]. Moreover, ROS can damage endoplasmic reticulum (ER) Ca^{2+} pumps, and sequentially cause Ca^{2+} leakage from ER, Ca^{2+} entrance to mitochondria (M), Ca^{2+}-induced cytochromec (CytC) leakage from mitochondria and, CytC-induced caspase (Casp) activation; these changes result in cell apoptosis and death.

system or the GTPases Ras1p and Ras2p. The GPCR would activate adenylate cyclase in response to high glucose levels. The function of Ras proteins might be related to the sensing and transmission of oxidative and other stress signals on the basis that the activity of the guanine nucleotide exchange factor for Ras, Cdc25p, is positively regulated by the heat shock protein Ssa1p (a member of the Hsp70 family) through a direct interaction. Specifically, it has been proposed that the accumulation of denatured proteins upon oxidative stress conditions would recruit Hsps in order to refold the proteins or lead them to a proteasome-dependent degradation, and thus, Hsps will not be available for activation of the cAMP-PKA pathway and cAMP, which is required for the transcription of G1 cyclins, will not be produced. This will result in G1-phase cell arrest (Igual and Estruch 2000; Crews 2003). Furthermore, upon activation of PKA the nuclear localization of the transcription factors, Msn2p and Msn4p, which induce the expression of H_2O_2-scavenging genes by binding to their stress response element (STRE) on DNA, is inhibited (Gorner and others 1998; Garreau and others 2000; Hasan and others 2002). By not allowing catalase, GPx, or other hydroperoxide-scavenging enzymes to be expressed, exogenous cAMP may inhibit differentiation by causing accumulation of H_2O_2 (and possibly LOOH), which is known at certain concentrations to sustain the undifferentiated state by promoting cell proliferation (Aguirre and others 2005). The outlined interrelated H_2O_2/cAMP signal transduction pathways in yeast need further exploration. Nonetheless, they can form a basis in understanding the corresponding pathways that govern the sclerotiogenic metamorphosis in filamentous fungi (Fig. 7). Besides their expected conservation throughout fungi, they may provide possible explanation for the observation that cAMP inhibits sclerotiogenesis in *S. sclerotiorum* and *R. solani* (Uno and others 1985; Rollins and Dickman 1998). Endogenous increase of cAMP (by exogenous sources) and the concomitant silence of antioxidant enzyme-encoding genes may result in $O_2^{\cdot-}$/H_2O_2 accumulation and decrease of sclerotiogenesis because of an extensive, not site specific, cell proliferation all over the fungal mycelial colony. This hypothesis is in agreement with unpublished data from our laboratory, which show that *S. sclerotiorum*, *R. solani*, *S. rolfsii*, and *S. minor* mycelial colonies undergo intense hyphal cell proliferation (they form undifferentiated thick mycelial colonies) when exposed to certain concentrations of H_2O_2 and cumene hydroperoxide; the latter is an artificial lipid hydroperoxide-analogue, suggesting a role for lipid hydroperoxides analogous to H_2O_2 in sclerotial metamorphosis.

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