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Review

Sclerotial development in *Sclerotinia sclerotiorum*: awakening molecular analysis of a "Dormant" structure

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ABSTRACT

Sclerotia are hard, asexual, resting structures which can survive for years in soil. In *Sclerotinia sclerotiorum*, which provides a good model system for studying sclerotial development, sclerotial development has been traditionally divided to three macroscopically distinct stages (initiation, development and maturation). However, additional phases (which can be visualized microscopically) indicate a complex, multi-step, process is involved. Environmental changes, primary metabolism and secondary messengers have been well documented factors affecting sclerotial development, yet analysis of the molecular mechanisms involved in sclerotial development is in its infancy. Here, we review the current status of the known molecular components involved in sclerotial development, with an emphasis on phosphorylative regulation of sclerotial development in *S. sclerotiorum*. Components such as cAMP-dependent protein kinase, ERK-like mitogen-activated protein kinase and Ser/Thr phosphatases type 2A and 2B, shown to regulate other developmental processes in fungi, have recently been shown to also be involved in regulation of sclerotium development. Reversible protein phosphorylation, as well as additional regulatory mechanisms of gene expression such as DNA methylation and ribosome inactivation, most likely function in concert with secondary metabolites, reactive oxygen species, pH and light in order to regulate sclerotial development in different fungi. The diversity of sclerotium-producing fungi promises to yield exciting variations into the molecular mechanisms regulating this developmental process in different species.

1. Introduction

Sclerotia are hard, asexual, resting structures composed of vegetative hyphal cells which become interwoven and aggregate together. It was only in the mid 19th century that these previously described structures were determined to be a true element of fungal origin (Léveillé 1843). Most studied filamentous fungi that produce sclerotia belong to a variety of genera that are members of the sub-phylums Ascomycotina (e.g., *Monilinia*, *Sclerotinia*, *Claviceps*, *Botrytis*, *Verticillium*, *Aspergillus* and *Penicillium*) or Basidiomycotina (*e.g., Typhula*, *Sclerotium* and *Coprinus*) (Willetts & Bullock 1992). Sclerotium formation

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has also been extensively studied in the myxomycete *Physarum polycephalum* (Chet & Henis 1975).

Sclerotia, as described in their landmark review by Willetts and Bullock (1992), usually consist of a continuous layer of pseudoparenchymatous, melanized cells known as a rind, which forms on the outer surface and encases a broad medulla of interwoven hyphae. In some sclerotia, a cortex of close-fitting hyphae can be distinguished immediately within the rind, grading into the more loosely interwoven hyphae of the medulla. An extracellular matrix usually accumulates around the medullary hyphae.

Sclerotium production is important in an economical context, as sclerotia are persistent resting and dissemination structures of some important agricultural crop pathogens, such as *Sclerotinia sclerotiorum, Sclerotium rolfsii, Botrytis cinerea* and *Claviceps purpurea*. In addition, the ergot alkaloids, which exhibit various pharmacological effects, are present in the sclerotia of some of the Claviciptaceae (Panaccione 2005; Tudzynski & Scheffer 2004).

In the last 50 y, research has focused mainly on sclerotial physiology and morphology, and on factors affecting sclerotial development and germination (Chet & Henis 1975; Punja 1985; Willetts & Bullock 1992, and recently reviewed by Georgiou *et al.* 2006). However, analysis of the molecular mechanisms involved in sclerotial development is still in its infancy. Functional and comparative genomic studies in plant pathogenic fungi have enormous potential to improve our understanding of the molecular mechanisms involved in developmental processes. The recent availability of the genome sequence of *S. sclerotiorum,* an important sclerotium-producing species (http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html), along with that of *B. cinerea* (http://www.broad.mit.edu/annotation/genome/botrytiscinerea/Home.html) and *Aspergillus flavus* (http://www.broad.mit.edu/annotation/genome/aspergillus_group/Regions.html/direct), provides an invaluable tool for hypothesis-driven research on sclerotium biology, as well as a basis for comparative analyses with other species. Identifying the molecular factors affecting sclerotial development and determining their roles will provide a better understanding of the basic morphological and physiological processes involved. In turn, this will enable us to control phytopathogenic fungi on the one hand, and improve the pharmaceutical exploitation of sclerotium-borne substances, on the other.

Here, we review the current status of known molecular components involved in sclerotial development, with an emphasis on phosphorylative regulation of sclerotial development in *S. sclerotiorum*.

S. sclerotiorum is a necrotrophic, phytopathogenic ascomycete with worldwide distribution which attacks more than 400 species of plants (Boland & Hall 1994; Purdy 1979; Tu 1997). Sclerotia of *S. sclerotiorum* can survive up to 8 y in soil (Adams & Ayers 1979) and can vary dramatically in size. On sunflower, for example, a sclerotium covering the seed layer may be 1 cm thick and exceed 3.5 cm in diameter, while on dry bean the sclerotia may be globose and 2 to 10 mm in diameter (Bolton *et al.* 2006). Sclerotia of *S. sclerotiorum* can germinate in two ways: carpogenically to form apothecia from which ascospores are liberated, or myceliogenically to produce hyphae (Abawi & Grogan 1979; Steadman 1979).

S. sclerotiorum provides a good model system for studying sclerotial development because: i) it produces multiple large sclerotia under standard laboratory conditions, ii) protoplast and *Agrobacterium*-mediated transformation and cloning of its genes by standard techniques is simple (Bolton *et al.* 2006; Weld *et al.* 2006 and references within), iii) its full genome sequence is now available, and iv) significant physiological and morphological analyses of this organism have been carried out over the last 50 y.

Sclerotial development has traditionally been divided to three distinct stages, based on macroscopically evident characteristics (Fig 1, upper panel): (1) initiation—the appearance of small distinct initial forms of interwoven hyphae (Fig 1A), (2) development—an increase in size (Fig 1B), and (3) maturation—characterized by surface delimitation, internal consolidation and pigmentation, and often associated with droplet secretion (Fig 1C; Townsend & Willetts 1954). Additional details of the sub-stages comprising the continuous process of sclerotium formation (e.g., initial hyphal densification, secretion along filaments, hyphal adhesion, etc.) can be observed microscopically (Fig 1, lower panels). The transition from linear hyphal growth to a more condensed sclerotium initial is accompanied by the secretion of a mucilage-like substance which may function as a hyphal adhesive (Fig 1D); this is followed by hyphal curling (Fig 1E), hyphal fusion (Fig 1F) and the formation of localized crystalline structures (Fig 1E,F) which may serve as a nucleus for the formation of the continuous outer sclerotium layer present in the mature sclerotium (Fig 1G). These islands of continuous secreted layers become wider and more condensed during the transition from white to mature black sclerotia (Fig 1H,I).

Hyphal branching and fusion

Hyphal branching and fusion are probably the main processes that occur once initiation is triggered. After the hyphal strands come into contact, they tend to adhere (Willetts & Bullock, 1992). The morphology of hyphal fusion between different strains of *S. sclerotiorum* was examined by Kohn *et al.* (1990), who observed anastomoses in some compatible interactions. Anastomosis occurred by direct hyphal fusion or was preceded by the winding of one hypha around the other or by the formation of a simple appressorium. In some pairings, anastomosis was followed by the formation of a cluster of hyphal initials from the point of fusion (Kohn *et al.* 1990), indicating the multiple cellular events that accompany this process. Hyphal fusion has been shown to be a complex phenomenon common to many fungal species and protein phosphorylation most likely plays a key role in its regulation (Glass *et al.* 2004), including during the onset of sclerotium formation. It would be interesting to see whether inter-strain hyphal fusion is mechanistically different from that which appears to occur during sclerotial formation (Fig 1F).

Melanization

A characteristic feature of sclerotial rind formation during the maturation phase is a gradual change of color from what is described by Willetts & Bullock (1992) as ''white to buff to dark brown or black'' resulting from the accumulation of melanin.

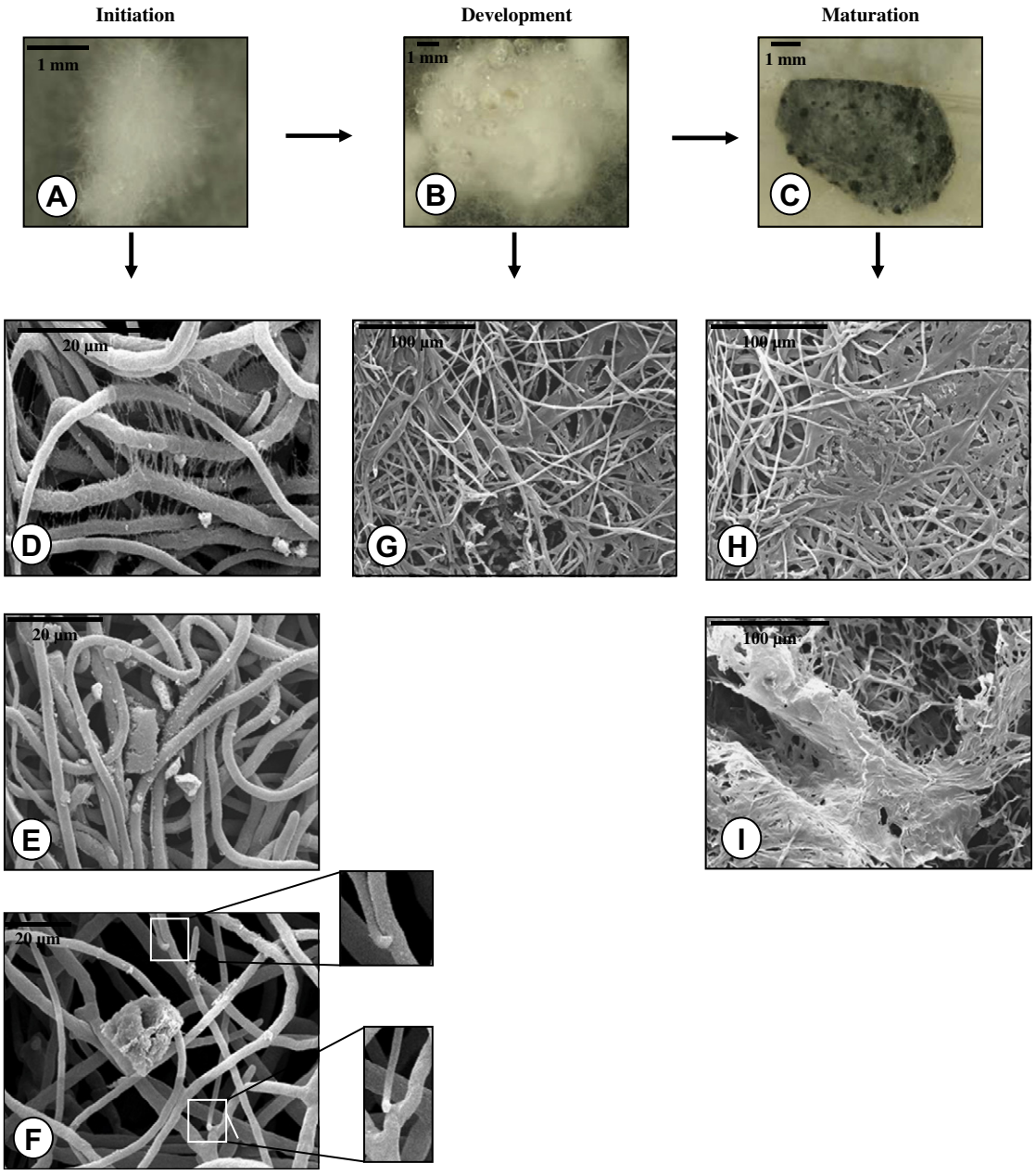

Fig. 1 – Macroscopic (upper panel) and microscopic (lower panels) development of *S. sclerotiorum* sclerotia. A. Initiation. B. Development. C. Maturation. D. Secretion of a mucilage-like substance. E. Curling of hyphae and formation of localized crystalline structures. F. Crystalline structures and hyphal fusion. G. Formation of a continuous outer sclerotium layer. H–I. A wider and more highly condensed secreted layer. For scanning electron microscopy, samples were fixed for 4 h with 5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. The samples were washed five times with the same buffer and then dehydrated in a series of 25 to 100 % ethanol washes (Yatzkan & Yarden 1999). The fixed samples were dried for 1 h in a CPD750 drier (Bio-Rad, Hercules, CA, U.S.A.) and gold-coated in a E5150 Polaron SEM coating system apparatus (Bio-Rad). The samples were observed under a Jeol (Tokyo, Japan) JSM 35 microscope.

The deposition of melanin in the rind walls in large amounts reduces cell permeability and protects the sclerotium from deleterious environmental agents, such as UV, reactive oxygen species (ROS) and biological degradation by enzymes produced by the fungus itself and/or by antagonistic microorganisms (Willetts & Bullock 1992). Melanin biosynthesis is also under the control of phosphorylative regulation, as recently demonstrated in *Cochliobolus heterostrophus*, which produces melanin *via* the 1,8 dihydroxynaphthalene (DHN) melanin biosynthesis pathway (Eliahu *et al.* 2007). Although fungal hyphae naturally synthesize DHN melanin (Henson *et al.* 1999), there is evidence that sclerotial melanin is produced through another pathway—the biosynthesis of L-dihydroxyphenylalanine (DOPA, an oxidation product of tyrosine)

(Chet & Henis 1969). In fungi, tyrosinases are mainly associated with browning and pigmentation (Halaouli *et al.* 2006) and their activity has been shown to correlate with an increase in sclerotial-initial formation in *S. sclerotiorum* (Wong & Willetts 1974). A tyrosinase-based melanin biosynthesis pathway in mamalian cells has been suggested to be affected by changes in protein phosphorylation mediated by Ser/Thr type-2A protein phosphatase (PP2A) and Mitogen Activated Protein Kinase (MAPK) activities (Kim *et al.* 2005). This may also prove to be the case in the regulation of sclerotial melanin production.

The multiple morphological changes that can be observed during sclerotial formation are regulated by a variety of external environmental cues. Some of the environmental factors affecting scleotia formation in a variety of species are described in the next part of this review. Realizing the assortment of the possible environmental factors involved is a significant step in linking external signals and/or conditions with the regulatory machinery involved in environmental signal perception and signal transduction (described further on) which affects sclerotial formation.

2. Environmental signals affecting sclerotial formation

Fungal morphogenesis, including propagule formation, is greatly influenced by the environment. Sclerotium formation is no exception. In this context, perhaps the most extensively studies sclerotium producing species is *Sclerotium rolfsii*. Chet and Henis (1975) reviewed common factors known to induce sclerotial formation in *S. rolfsii*, including light, temperature, pH, oxygen, CO_2 and mechanical factors. Sclerotial formation in *S. rolfsii* can also be induced by amending the growth medium with iodoacetic acid (Chet *et al.* 1966), lactose (Okon *et al.* 1972) or threonine (Kritzman *et al.* 1976), or by transferring the mycelium from a submerged to aerial growth environment (Hadar *et al.* 1981). *S. sclerotiorum* does not always respond similarly to these inducing factors (Erner and Yarden, unpublished). However, shearing *S. sclerotiorum* mycelium and subsequently transferring it from submerged culture to solid medium induces the near-synchronous formation of sclerotia (Harel *et al.* 2005), indicating that mechanical disruption along with desiccation can induce sclerotium formation in this species.

Light

Light can affect fungal development, in some cases *via* the activity of circadian clocks (Liu & Bell-Pedersen 2006). Although rhythms in fungal-spore development and discharge are widespread, information concerning their involvement in sclerotial development is scarce. Involvement of circadian oscillators in sclerotial development among different fungal species is apparently not uniform. The *A. parasiticus veA* gene and its homologue in *A. flavus* have been shown to be involved in sclerotial formation (Calvo *et al.* 2004; Duran *et al.* 2006). As *veA* is known to mediate a developmental light response in *A. nidulans* (Yager 1992), it is tempting to speculate that *veA* is also involved in sclerotial development *via* the circadian-clock system in the sclerotium-producing *Aspergilli*. Nonetheless, we have not detected circadian oscillation in sclerotium formation in *S. sclerotiorum* (Erental and Yarden, unpublished).

pH

In general, the most favorable pH for mycelial growth is also optimal for sclerotial formation (Rudolph 1962; Townsend 1957). Neutral or alkaline pH levels have been shown to inhibit sclerotial formation in *S. sclerotiorum*, whereas oxalic acid accumulation lowers the ambient pH, creating conditions which favor sclerotial development (Rollins & Dickman 2001). Furthermore, *S. sclerotiorum* mutants deficient in oxalic acid production were also unable to produce sclerotia (Godoy *et al.* 1990) suggesting that low pH may play a role in the process of sclerotial development in this species. In *Aspergillus nidulans*, many gene products with pH-sensitive activities have been shown to be regulated by a common transcription factor - PacC (Caddick *et al.* 1986). Rollins (2003) functionally characterized a *pacC* gene homologue, *pac1*, from *S. sclerotiorum* and produced mutants with loss-of-function alleles in the *pac1* locus by targeted gene replacement. Sclerotial development and maturation in culture was aberrant in these *pac1*-replacement mutants and the mutant failed to produce melanized outer rinds (Rollins 2003).

Secondary metabolism

Secondary metabolism has been clearly shown to be associated with fungal development. For example sclerin, a secondary phenolic metabolite which is produced by *S. sclerotiorum* and *Sclerotinia minor*, has been shown to stimulate the activities of polyphenoloxidase (see below) and peroxidase and to induce the formation of initials and melanogenesis in sclerotia (Marukawa *et al.* 1975). These results are in line with the accumulating genetic evidence linking secondary-metabolite production with different developmental processes (Yu & Keller 2005). In addition, *S. sclerotiorum* mutants deficient in the production of another secondary metabolite (which in a different fungus - *Fomitopsis palustris* has, nevertheless, been linked with primary metabolic cycles; Munir *et al.* 2001) and key pathogenicity factor - oxalic acid, are also unable to produce sclerotia (Godoy *et al.* 1990). Thus, secondary-metabolite production is either important for sclerotial development or is co-regulated with this developmental process for reasons that have yet to be elucidated. A similar phenomenon has been found in *A. parasiticus* and *A. flavus*, where deletion of the *veA* gene resulted in the complete blockage of sclerotial formation as well as blocked production of mycotoxin intermediates (Calvo *et al.* 2004; Duran *et al.* 2006).

Reactive oxygen species (ROS)

In all eukaryotes examined to date, ROS are produced during normal cellular metabolism. It is now evident that low, nonlethal, concentrations of ROS can function beneficially as regulatory molecules in cell-signalling pathways. The importance of the redox ''climate'' in fungal growth and development has been suggested by Hansberg and Aguirre (1990),

who proposed that hyperoxidant states are a primary driving force for the differentiation states in micro-organisms, including fungi (Fang et al. 2002; Lara-Ortiz et al. 2003). Georgiou et al. (2006) summarized the evidence implying that sclerotial differentiation is affected by oxidative stress as well, as exemplified by the fact that a variety of hydroxyl radical scavengers inhibit sclerotial differentiation and growth of *S. sclerotiorum* and *Rhizoctonia solani* (Georgiou et al. 2000). Additional evidence for the involvement of ROS in sclerotial metamorphosis (in *S. rolfsii*, *R. solani*, *S. sclerotiorum* and *S. minor*) is provided by the decrease in sclerotial numbers in cultures exposed to the O_2 - scavenger Tempol, a mimetic of the antioxidant enzyme SOD (Georgiou et al. 2006). The role of ROS as signal molecules during sclerotial development has yet to be elucidated, but clear indications as the involvement of ROS in this process is emerging (see below).

Environmental perception is a key attribute required for survival and development in any organism and fungi are no exception. The fact that filamentous fungi possess a large number of sensory components (Borkovich et al. 2004) emphasizes this fact and establishes the significance of linking external stimuli with the internal regulatory networks and downstream components governing growth and development.

3. Cellular sensing and signal transduction

G protein coupled receptors (GPCRs), adenylate cyclase and cAMP

GPCRs are key links between the environment and the regulatory machinery residing within the cell and their implication in sclerotial development has been demonstrated. One established role of the Gα subunit of the heterotrimeric G protein is the regulation of adenylate cyclase activity. A *B. cinerea* (closely related to *S. sclerotiorum*) Δ*bcg3* mutant, disrupted in the Gα3 subunit, produced a large number of sclerotia under conditions which do not lead to sclerotium formation in the wild type, as a result of the reduction in cAMP levels. Addition of cAMP to the growth medium suppressed sclerotium formation in the Δ*bcg3* mutant in a manner similar to that observed in the wild type (Doehlemann et al. 2006), implying that cAMP negatively regulates sclerotial development in *B. cinerea*.

The enzyme adenylate cyclase is activated by Gαs-GTP and synthesizes the second messenger cAMP from ATP. To determine the biological roles of cAMP, a key factor affecting many developmental processes in fungi (Lee et al. 2003 and references within), in *S. sclerotiorum*, Jurick and Rollins (2007) decreased intracellular cAMP by disrupting the single-copy adenylate cyclase gene (*sac1*) from *S. sclerotiorum*. In the *sac1*-disrupted mutant, hyphal branching was altered, microconidia (spermatia) were more abundant, and aberrant sclerotia were produced in an abnormal manner. As *S. sclerotiorum* typically forms sclerotia at the outer edge of plates, the fact that the *S. sclerotiorum sac1* mutant produced sclerotia without reaching the edge of the plate is consistent with Rollins and Dickman (1998) observations which demonstrated an inhibitory role for cAMP in regulating sclerotial initiation. In contrast, high intracellular cAMP levels have been shown to induce sclerotium initiation in *Rhizoctonia solani* (Hashiba & Ishikawa 1978) and *S. rolfsii* (Hadar et al. 1983). Moreover, addition of exogenous cAMP to the growth medium of a wild-type *R. solani* isolate increased sclerotial development (Rollins & Dickman 1998). Thus, cAMP most likely plays a role in sclerotial formation, but may do so differently in different species.

Hyphal branching and fusion are probably the main processes that occur once initiation is triggered. The role of cAMP in sclerotial initiation likely involves changes in hyphal elongation/branching rates. This is in line with the fact that low intracellular cAMP levels have been shown to accompany increased branching rates and decreased rate of radial expansion in *Neurospora crassa* (Rosenberg & Pall 1979). A link between hyphal branching and sclerotial morphogenesis has also been established in *S. rolfsii* (Goujon 1970; Henis et al. 1973). The fact that in the *S. sclerotiorum sac1* mutant (in which cAMP levels are low and sclerotial formation is abnormal) hyphal-branching is impaired suggests the presence of a link between levels of the secondary messenger, branching and proper sclerotial formation.

A few of the sclerotia produced by the *S. sclerotiorum sac1*-disrupted strain were capable of myceliogenic germination; however, they did not produce apothecia (Jurick & Rollins 2007). This implies that the mechanisms governing these two germination pathways are different. Another line of evidence supporting the mentioned distinction can be found in the results obtained by the analysis of a sclerotium-specific protein. Sclerotia of *S. sclerotiorum* contain a major protein (~35–40 % of the mature sclerotial protein content) of about 36,000 daltons which is not detected in vegetative cells. The abundance of this protein does not decrease in sclerotial residue or appear in vegetative cells when sclerotia germinate myceliogenically. In contrast, the abundance of this protein does decrease in sclerotia undergoing carpogenic germination and a small amount of the protein is present in the resultant apothecia (Russo et al. 1982). Thus, it appears that myceliogenic and carpogenic germination pathways in *S. sclerotiorum* can be governed by different mechanisms.

PKA

cAMP is a key modulator of cAMP-dependent protein kinase A (PKA) activity. Relative PKA activity increased during the white sclerotium stage in a wild-type *S. sclerotiorum* strain, while low levels were maintained in non-sclerotium-producing mutants, strongly suggesting that PKA activity is involved in sclerotial development (Harel et al. 2005). Furthermore, applying caffeine, known to induce PKA activity (yet also affects other cellular components), increased relative PKA activity levels and was correlated with the formation of sclerotial initial-like aggregates in cultures of the non-sclerotium-producing mutants. The observation that caffeine induces hyphal fusion in the filamentous ascomycete *N. crassa* raised the possibility of this also being the case in *S. sclerotiorum* (Harel et al. 2005). Thus, PKA may also be involved in the early developmental stage of hyphal fusion. In addition, the antisense *smk1* strain (see below), which did not produce sclerotia, exhibited reduced PKA activity (Harel et al. 2005). In contrast, mutants deficient in a *S. sclerotiorum* PKA catalytic subunit gene, *pka1* (SS1G_03171.1), produced sclerotia, were cAMP-responsive

and pathogenic, and exhibited wild-type levels of PKA activity (Jurick *et al.* 2004). This apparent contradiction can be reconciled by the notion that a gene encoding a second PKA catalytic subunit, *pka2* (SS1G_01124.1), contributes sufficient PKA activity in *S. sclerotiorum*.

It is likely that the role of PKA (and components of other signal transduction pathways) in sclerotial development will prove to be complex. The fact that PKA function is concerted with additional signal transduction pathways (Kronstad *et al.* 1998; Lengeler *et al.* 2000; Stork & Schmitt 2002) poses a challenge in their dissection. Furthermore, recent evidence point to the possibility that PKA is not only responsible for protein phosphorylation, but can also play a part in the regulation of dephosphorylation events *via* its activity on a subunit of type 2A phosphatase (Ahn *et al.* 2007), which has also been shown to play a role in sclerotium formation (see below). A model describing some of the signal transduction components proposed to be involved in sclerotial development in *S. sclerotiorum* can be found in Fig 2.

The Ras-MAPK signalling pathway

Extracellular stimuli lead to activation of a MAP kinase *via* a signaling cascade (“MAPK cascade”) composed of MAP kinase, MAP kinase kinase, and MAP kinase kinase kinase. MAPK is activated by phosphorylation of conserved tyrosine and threonine residues by a unique and dedicated, dual specificity MAPK kinase (MAPKK or MEK). In turn, the MAPKK is activated by phosphorylation on conserved serine and threonine residues by the MAP kinase kinase kinase (MAPKKK or MEKK). MAP kinases become enzymatically activated by phosphorylation in response to various extracellular stimuli and for that reason are also termed ERKs (extracellular signal regulated kinases). In filamentous ascomycetes, such as *Neurospora crassa*, three MAP kinases have been identified, which are homologues of the yeast MAP kinases Fus3/Kss1, Hog1 and Slt2, (Galagan *et al.* 2003). The Slt2 MAPK homologue in filamentous fungi has been shown to be involved in the maintenance of cell-wall integrity and various aspects of saprotrophic and pathogenic growth (Hou *et al.* 2002; Kojima *et al.* 2002; Mehrabi *et al.* 2006). Chen *et al.* (2004) demonstrated that the homologue of an ERK-type mitogen-activated protein kinase in *S. sclerotiorum* (Smk1) is required for sclerotial development. *smk1* transcription and MAPK enzyme activity are dramatically induced during sclerotiogenesis, especially during the production of initials. When a specific inhibitor of MAPK activity was applied to cultures or when antisense expression of *smk1* was induced, sclerotial maturation was impaired, implying that Smk1 is involved in subsequent development stages as well. In addition, *smk1* transcript levels were found to be highest under acidic pH conditions, suggesting that Smk1 regulates sclerotial development *via* a pH-dependent signalling pathway involving the accumulation of oxalic acid. A *B. cinerea* Δ*bmp1* MAPK mutant did not form sclerotia either, suggesting that regulation of sclerotium formation in this species is at least partially similar to that observed in *S. sclerotiorum* (Doehlemann *et al.* 2006). Furthermore, the Slt2-type MAPK (Bmp3) is also required for sclerotial formation in *B. cinerea* (Rui & Hahn 2007). Addition of cAMP inhibited *smk1* transcription, MAPK activation, and sclerotial development in *S. sclerotiorum*. Thus, *S. sclerotiorum* can respond to environmental signals (such as pH) and most likely even coordinate them to trigger a signalling pathway mediated by Smk1 to induce sclerotial formation, one which is negatively regulated by cAMP (Chen *et al.* 2004).

Even though cAMP is a known activator of PKA, evidence has been provided for the presence of a novel mechanism by which cAMP activates the MAPK pathway in a PKA-independent manner (Chen & Dickman 2005). These authors showed that inhibition of Ras, an upstream activator of the MAPK pathway, inhibits sclerotial development and MAPK activation, suggesting that a conserved Ras/MAPK pathway is required for sclerotial development. They also showed that applying a Rap-1 inhibitor restores MAPK activation in the presence of cAMP. The fact that inhibition of Rap-1 can restore cAMP-blocked sclerotial development further supports Rap-1’s potential role in cAMP-dependent MAPK inhibition (Chen & Dickman 2005). Nonetheless, these results do not rule out the possibility that sclerotial development is mediated by both PKA-dependent and PKA-independent processes.

All MAPK pathways operate through sequential phosphorylation events to phosphorylate transcription factors and regulate gene expression. It will be interesting to examine whether the *pac1* transcription factor activity is under the control of Smk1 as well.

4. Involvement of protein phosphatases in sclerotial development

As protein phosphatases (PPs) are an integral part of the machinary regulating reversible phosphorylation, they are key elements in maintaining the balance of many cellular activities. However, the study of their involvement in sclerotial development has only recently been initiated. On the basis of substrate specificity, PPs are classified as Ser/Thr, Tyr, dual-specificity or His PPs, and representatives of PP subgroups have been identified in filamentous fungi (Borkovich *et al.* 2004; Dickman & Yarden 1999).

PP2B

Harel *et al.* (2006) demonstrated the importance of type 2B phosphatase (PP2B or calcineurin) in sclerotial maturation, by showing alterations in its expression in *S. sclerotiorum* in a phase-specific manner during sclerotial development. The highest increase in PP2B expression levels was observed in mature sclerotia, suggesting its multiple regulatory functions in sclerotial development. Inhibition of PP2B by FK506, cyclosporin A, or inducible antisense calcineurin expression impaired sclerotial maturation (Harel *et al.* 2006). In parallel, Rodriguez *et al.* (2006) showed that when the culture filtrate of a cyclosporin-A-producing strain of *Fusarium oxysporum* is added to *S. sclerotiorum* media, there is a significant reduction in the number of sclerotia produced and their average dry weight is significantly higher. White structures resembling immature sclerotia were also produced. In yeast, dephosphorylation of the Zn-finger transcription factor Crz1p

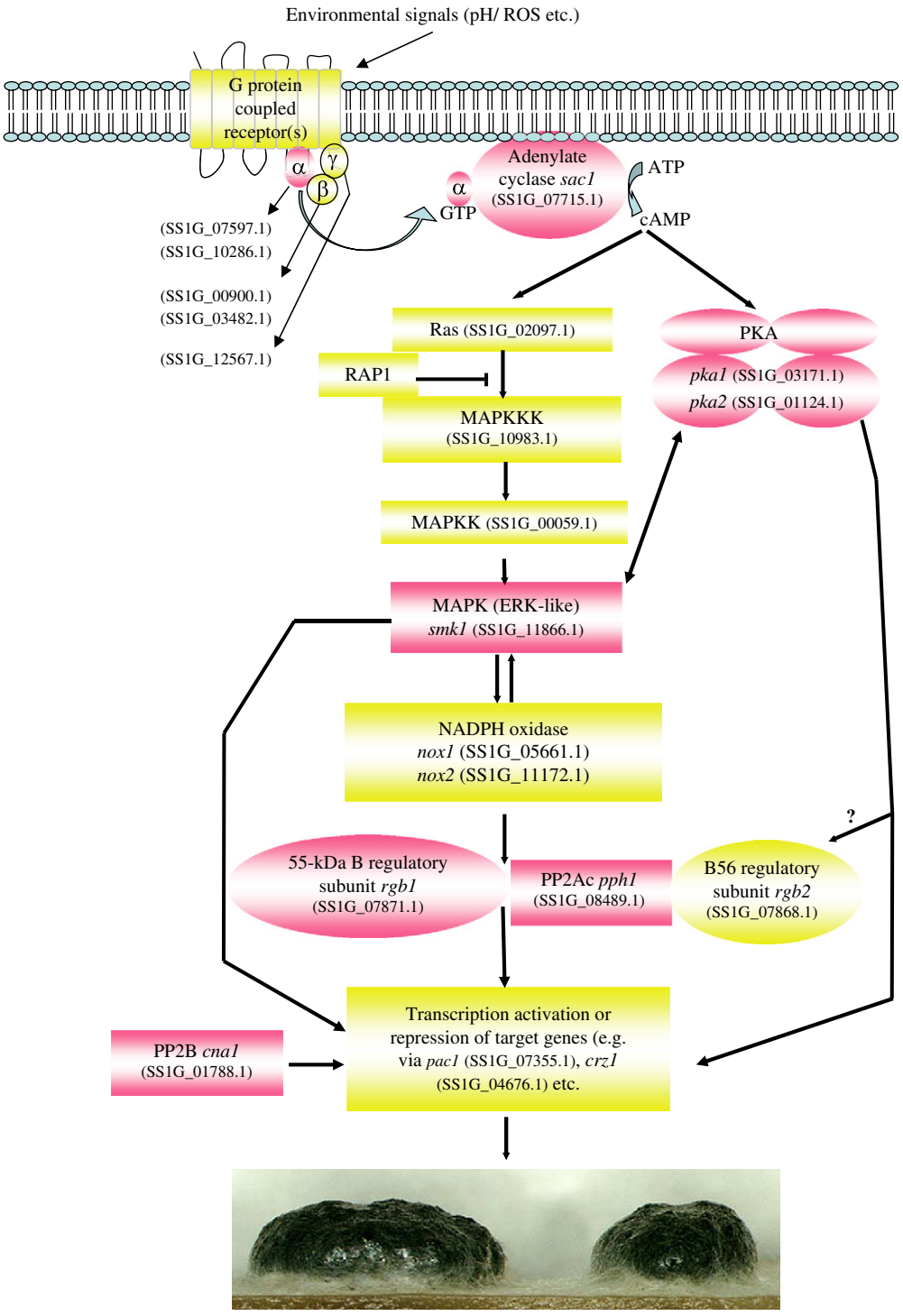

Fig. 2 – Model summarizing signal transduction components (with an emphasis on those analyzed to date – marked in red) proposed to be involved in sclerotial development in *Sclerotinia sclerotiorum*. Some of the hierarchical positioning of the different components and the nature of their interactions (be them positive, negative or cooperative regulatory effects) have yet to be fully resolved. Details are discussed in the text. Numbers in parentheses indicate current genome annotation references as appear at http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html.

by calcineurin results in translocation of Crz1p to the nucleus where it activates expression of genes (Stathopoulos-Gerontides *et al.* 1999). This transcription factor has also been shown to be phosphorylated by PKA, thus linking PKA and PP2B signaling (Kafadar & Cyert 2004). Nevertheless, the details of PKA and PP2B-based regulation of Crz1 activity (be it cooperatively or in opposing manners) has yet to be elucidated. Evidence has also been produced for the existence of

a calcineurin-MAPK-associated pathway. For example, in human cells, transcriptional activity of NFATc2 (a Crz1p homolog) is upregulated by phosphorylation of the MAPK JNK (Ortega-Perez *et al.* 2005). It will be interesting to examine whether the putative Crz1p homologue in *S. sclerotiorum* (SS1G_04676.1) is involved in sclerotial formation and whether it functions *via* the activity of PP2B and/or MAPK pathways.

Transcripts of *cna1* (encoding PP2B) were found to accumulate in sclerotia of *S. sclerotiorum*. In apparent contrast, inhibition of PP2B by a pharmacological approach or inducible antisense *cna1* expression increased germination of preformed sclerotia (Harel *et al.* 2006). Opposing mechanistic explanations for this observation can be suggested: on the one hand, active PP2B may be required in mature sclerotia for the inhibition of untimely germination (hence, inhibition of PP2B would trigger the germination process); on the other, an abundance of PP2B mRNA may be required during the early stages of germination, and it therefore accumulates in the sclerotia. Indeed sclerotia of *P. polycephalum* have been shown to store mRNA sequences in association with a distinct set of proteins (Adams *et al.* 1981). The latter explanation is supported by the fact that a ribosome-inactivating protein, pleuturegin, which inhibits translation in a cell-free rabbit reticulocyte lysate system, was purified from fresh sclerotia of another fungus—the edible mushroom *Pleurotus tuber-regium* (Wang & Ng 2001). This implies that at least in that species, stored mRNA cannot be translated in dormant sclerotia when such proteins are present. Even though only a few ribosome-inactivating proteins have been described in fungi, with an emphasis on those that are secreted (Ng 2004), it is likely that such proteins are involved in translation regulation within the sclerotium.

PP2A

PP2A, an additional Ser/Thr PP, has been recently shown to be required for sclerotial development: in strains in which production of the variable B regulatory subunit of PP2A was reduced, sclerotial maturation was severely impaired (Erental *et al.* 2007). Furthermore, even though different cellular components can affect PP2A activity, experimental evidence demonstrates that PP2A activity in *S. sclerotiorum* is dependent on the function of both Smk1 (described earlier) and the superoxide-producing NADPH oxidase (Nox). Nox activity has been shown to decrease in the *smk1* mutant (Erental *et al.* 2007). In the same manner, research in mammalian cells has provided evidence that Nox expression is activated by the MAPK pathway (Mitsushita *et al.* 2004). The latter finding concurs with the hypothesis that ROS play a key role in the signalling of sclerotial development in filamentous fungi (Georgiou *et al.* 2006). As PKA and MAPK are involved in sclerotial development, and PPs play an important role in maintaining the balance of the phosphorylation-regulation machinery, the crucial role played by PPs in sclerotial development is not surprising. On the contrary, the development of sclerotia is probably dependent on the concerted activity of upstream-signal and downstream-effected molecules along with specific kinases and phosphatases.

5. Conclusions

Even though sclerotium production is an integral part of the life cycle of a variety of fungi and can be highly significant in an economical context, analysis of the molecular mechanisms involved in sclerotial development is in its infancy. The evidence that has been accumulated indicates that the mechanisms governing sclerotial development include components involved in reversible phosphorylative regulation, which is known to coordinate other phases of fungal growth, proliferation and morphogenesis. In addition, it is now becoming clear that primary and secondary metabolism, along with ROS, all play significant roles in sclerotial development. In this article, we chose to focus on sclerotial formation in *S. sclerotiorum*. As many fungi have the same basic sclerotial structure, anatomy, physiology and biochemistry, it is reasonable to assume that some of the molecular modules regulating sclerotial formation will be similar as well, even though their mode of action may differ (as is the case of cAMP). On the other hand, and on the basis of the diverse genera of sclerotium-producing fungi, we can also expect to find some major differences. Perhaps an analogy can be found in the case of conidiation in *A. nidulans* and *N. crassa*: significant conservation among the upstream signalling components regulating conidiation reflects the similar ways in which these fungi respond to the same environmental signals to initiate macroconidial development; on the other hand, there appears to be a lack of conservation in the downstream signalling components, suggesting that conidiation has evolved independently in these two organisms (Borkovich *et al.* 2004). Elucidation of the complex activities leading to sclerotial formation, the activities within the resting structure and germination is still a long way off. Nevertheless, the rapid accumulation of reports in the past few years is clear testimony to the fact that the molecular analysis of sclerotial biology is far from dormant.

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