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MAPK Regulation of Sclerotial Development in *Sclerotinia sclerotiorum* Is Linked with pH and cAMP Sensing

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Sclerotial development is fundamental to the disease cycle of the omnivorous broad host range fungal phytopathogen *Sclerotinia sclerotiorum.* We have isolated a highly conserved homolog of ERK-type mitogen-activated protein kinases (MAPKs) from *S. sclerotiorum* (Smk1) and have demonstrated that Smk1 is required for sclerotial development. The *smk1* transcription and MAPK enzyme activity are induced dramatically during sclerotiogenesis, especially during the production of sclerotial initials. When PD98059 (a specific inhibitor of the activation of MAPK by MAPK kinase) was applied to differentiating cultures or when antisense expression of *smk1* was induced, sclerotial maturation was impaired. The *smk1* transcript levels were highest under acidic pH conditions, suggesting that Smk1 regulates sclerotial development via a pH-dependent signaling pathway, involving the accumulation of oxalic acid, a previously identified pathogenicity factor that functions at least in part by reducing pH. Addition of cyclic AMP (cAMP) inhibited *smk1* transcription, MAPK activation, and sclerotial development. Thus, *S. sclerotiorum* can coordinate environmental signals (such as pH) to trigger a signaling pathway mediated by Smk1 to induce sclerotia formation, and this pathway is negatively regulated by cAMP.

Additional keywords: morphogenesis; signal transduction.

Sclerotinia sclerotiorum is among the world's most successful and omnivorous fungal phytopathogens, able to infect an extremely wide range of cultivated plants (Boland and Hall 1994; Purdy 1979). The dispersal, propagation, and long-term survival of this pathogen is mediated through the sclerotiorum, a pigmented, multihyphal structure which is able to remain quiescent for extended periods under conditions that are unfavorable for vegetative growth (Chet and Heneis 1975; Willetts and Bullock 1992; Willetts and Wong 1980). The mechanisms for inducing and regulating sclerotiorum development are being investigated. Previous studies (Rollins and Dickman 1998, 2001) showed that alterations in environmental pH, as well as cyclic AMP (cAMP) levels, were implicated as primary cues in triggering sclerotiorum development.

Oxalic acid (OA) has been suggested to be an essential pathogenicity determinant in *S. sclerotiorum* because mutants deficient in OA production were nonpathogenic (Dickman and Mitra 1992; Godoy et al. 1990). Interestingly, such OA^- mutants also are unable to develop sclerotia and a revertant to OA^+ also then produced sclerotia (Godoy et al. 1990; Rollins and Dickman 1998). Culture medium pH has been shown to significantly influence OA biosynthesis and sclerotial development in an inverse relationship (Maxwell and Lumsden 1970; Rollins and Dickman 2001; Vega et al. 1970). Neutral or alkaline pH rapidly increased OA accumulation but suppressed sclerotial development. OA accumulation leads to the lowering of ambient pH and forms conditions that favor sclerotial development (Rollins and Dickman 2001).

These findings suggest that a pH-mediated signaling pathway might function to regulate OA production and sclerotial development. A key component in fungal pH signaling cascades is pacC/Rim101p, a zinc finger transcription factor that activates alkaline-expressed genes and represses acid-induced genes in a number of fungi, particularly *Aspergillus nidulans*, where the bulk of such studies has been performed (Arst and Penalva 2003; Caracuel et al. 2003; Espeso and Arst 2000; Lamb and Mitchell 2003; Lamb et al. 2001; MacCabe et al. 1996; Ramon et al. 1999; Tilburn et al. 1995). Moreover, the products of the six pH signal transduction pathway (*pal*) genes are required for the proteolytic processing of PacC to yield its functional form (Arst et al. 1994; Caddick et al. 1986; Mingot et al. 1999; Negrete-Urtasun et al. 1999; Orejas et al. 1995). Pac1, a functional homolog of *A. nidulans* PacC, was identified in *S. sclerotiorum* and demonstrated to be involved in sclerotial development and pathogenesis (Rollins 2003; Rollins and Dickman 2001) Similar to PacC, *pac1* displayed pH-regulated expression with peak expression under neutral or alkaline pH conditions. However, with the exception of Pac1, the involvement of other effectors in pH-mediated signaling, such as *A. nidulans pal* genes, currently is unknown in *S. sclerotiorum.*

cAMP has been shown to play an important role in regulating a variety of processes in fungi, including differentiation, sexual development, pathogenicity, and the monitoring of environmental stresses (Kronstad et al. 1998; Kronstad 2000). In the basidiomycete *Ustilago maydis,* cAMP signaling controls the transition between budding and filamentous growth (Gold et al. 1994). Mutations in *uac1,* an adenylyl cyclase-encoding gene, or *adr1,* a gene encoding the catalytic subunit of cAMP-dependent protein kinase (PKA), both caused constitutively filamentous growth (Durrenberger et al. 1998; Gold et al. 1994). Moreover, the cAMP/PKA pathway plays an important role in regulating the transition between vegetative growth and conidiation, and is essential for pathogenic development

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in the filamentous phytopathogen *Colletotrichum trifolii* (Yang and Dickman 1997, 1999a,b). cAMP signaling also has been investigated in *S. sclerotiorum;* Rollins and Dickman found that cAMP plays a role in the regulation of the transition between mycelial growth and sclerotial development. Increases in endogenous or exogenous cAMP levels inhibited sclerotial development while substantially elevating OA accumulation (Rollins and Dickman 1998). Considering the role of environmental pH in OA accumulation and sclerotial development, it is possible that cAMP-mediated signaling may interact with or function in parallel to pH signaling to regulate OA levels and sclerotial development.

The mitogen-activated protein kinase (MAPK) cascade has been studied in an expanding number of filamentous fungi, including *Magnaporthe grisea* (Xu and Hamer 1996), *Cochliobolus heterostrophus* (Lev et al. 1999), *Colletotrichum lagenarium* (Takano et al. 2000), *U. maydis* (Mayorga and Gold 1999; Muller et al. 1999), *Botrytis cinerea* (Zheng et al. 2000), and *Fusarium oxysporum* (Di Pietro et al. 2001). The highly conserved MAPK modules have been reported to be involved in regulating several essential developmental processes in fungi, such as sporulation, mating, hyphal growth, and pathogenicity (Dickman and Yarden 1999; Xu 2000). In addition, MAPK signaling cascades were found to function coordinately with other pathways, such as cAMP/PKA and pH-dependent pathways in fungi (e.g., *Saccharomyces cerevisiae, Schizosaccharomyces pombe*, *Candida albicans*, and *F. oxysporum*) during development and response to environmental stresses (Caracuel et al. 2003; Kronstad et al. 1998; Leberer et al. 2001).

Because pH and cAMP are involved in the regulation of sclerotial morphogenesis, we asked whether pH-dependent and cAMP-dependent signaling pathways are components of a common signaling circuit or whether they operate through independent pathways but regulate common downstream components. In the present study, we identify an ERK-type MAPK homologue (*smk1*) in *Sclerotinia sclerotiorum* and investigate the role of Smk1 in sclerotial development. Our results provide evidence that Smk1, a highly conserved MAPK homolog in *S. sclerotiorum*, functions to regulate sclerotial development through interconnections with pH-dependent and cAMP-dependent pathways.

RESULTS

Inhibition of MAPK activation interferes with sclerotial development.

Recent studies have indicated that MAPKs are highly conserved and play key roles in regulating mating, morphogenesis, virulence, and stress responses in many filamentous fungi (Dickman and Yarden 1999). In an attempt to explore the contribution of MAPK activation to the growth and development of *S. sclerotiorum*, we treated the wild-type *S. sclerotiorum* isolate 1980 with 10 μM PD98059, a specific MAP kinase kinase (MEK) inhibitor (Alessi et al. 1995; Pang et al. 1995). The treatment of cells with PD98059 delayed sclerotial maturation (Fig. 1), suggesting that MEK-dependent activation of MAPK is required for sclerotial development.

Isolation of the *S. sclerotiorum smk1* gene.

We isolated a MAPK homologue (*smk1*) from *S. sclerotiorum* by performing a polymerase chain reaction (PCR)-based library screening strategy. The open reading frame of *smk1* is predicted to encode a 356-amino acid protein (Smk1) with 94, 91, 91, 92, and 58% identity with *Botryotinia fuckeliana* BMK1 (AF205375), *F. oxysporum* FMK1 (AF286533), *M. grisea* PMK1 (AACU01000997), *Colletotrichum lagenarium* CMK1 (AF174649), and *Mus muslucus* ERK2 (P27703), respectively (Fig. 2A). The putative *smk1* coding region contains all the consensus motifs present in MAPKs, including the characteristic MAPK phosphorylation sites (TEY, residues 183 to 185). Southern analysis of genomic DNA digested with different restriction enzymes and probed with *smk1,* under high stringency, revealed that this is a single-copy gene in the *S. sclerotiorum* genome (data not shown).

smk1 transcript abundance and levels of phosphorylated MAPK are increased during initiation of sclerotial development.

We examined the abundance of *smk1* transcript levels during various stages of fungal growth and development. Cultures grown on potato-dextrose agar (PDA) medium were harvested separately during different stages of sclerotiogenesis (initial sclerotia, developing sclerotia, and mature sclerotia). Northern analysis showed that *smk1* transcript levels were highest during the sclerotia initials-forming stage (Fig. 2B, lane 1).

We next tested whether the increase in *smk1* transcript abundance is accompanied by changes in MAPK activity levels. The activation of MAPK was determined by analyzing the phosphorylation of MAPK in protein extracts of sclerotia-producing cultures, using the antiphosphorylated ERK antibodies (which were chosen on the basis of the high sequence similarity between Smk1 and ERK). Based on our results (Fig. 2C), a specific and substantial increase in phosphorylated (thus active) MAPK was evident during the initiation phase of sclerotial production. Additional evidence demonstrating the detection of phosphorylated MAPK by these antibodies was provided by analysis of protein extracts obtained from strains expressing antisense *smk1* (discussed below).

Expression of an antisense *smk1* severely affects fungal growth and development in *S. sclerotiorum*.

The results obtained when applying the MEK inhibitor, coupled with increases in *smk1* transcript levels and activated MAPK enzyme levels during the initiation of sclerotial formation, suggest that Smk1 may play an important role in regulating sclerotial development. To test this hypothesis, a construct expressing an antisense *smk1* (where an inverted *smk1* cDNA was cloned downstream of the conditional *qa-2* promoter from *Neurospora crassa*, in the pSO-1 vector) was inserted into the wild-type strain and the effect of *smk1* antisense expression on fungal morphology was determined. When *smk1* antisense expression was induced, sclerotial maturation and hyphal growth were severely blocked (Fig. 3A). To confirm the correlation between the phenotype observed and the abundance of *smk1* transcript levels and also to investigate the effect of quinic

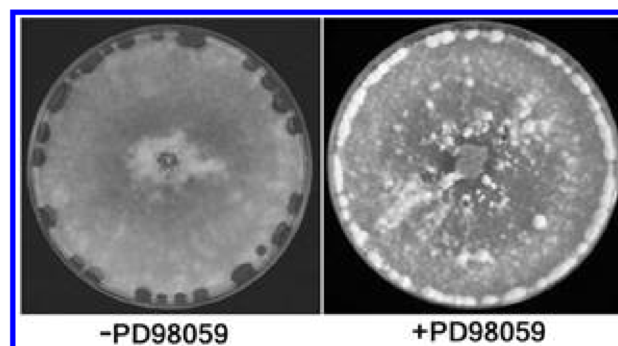

Fig. 1. Inhibition of sclerotial maturation in *Sclerotinia sclerotiorum* 1980 cultures grown on potato-dextrose agar (PDA) supplemented with 10 μM PD98059. Dishes were inoculated, in the center, with a small agar-mycelium plug from a 5-day-old PDA culture. The cultures were photographed 10 days postinoculation.

acid-induced antisense *smk1* expression under ambient pH condition, Northern analyses were performed on extracts from mycelia of the *smk1* antisense mutants. Results of our analysis (Fig. 3B) demonstrate that the *smk1* transcript was detected in RNA isolated from the antisense cassette-harboring strain cultured in minimal media amended with 15 mM glucose (where the *qa-2*-driven *smk1* antisense expression was repressed) but not when the medium was amended with 15 mM quinic acid, where the *qa-2*-driven *smk1* antisense expression was induced.

To verify that, in addition to transcript abundance, expression of *smk1* antisense also affected levels of phosphorylated MAPK, we extended our analysis to include immunodetection of the phosphorylated MAPK. As expected, the reduction in *smk1* transcript abundance was accompanied by reduced levels of detectable phosphorylated MAPK when the *qa-2*-driven *smk1* antisense expression was induced by quinic acid (Fig. 3C). These results also confirm that the anti-ERK antibodies detect the fungal MAPK protein. Taken together, these findings strongly support the notion that MAPK plays an essential role in regulating fungal growth and sclerotial development.

smk1 expression is pH regulated.

Rollins and Dickman (2001) previously have shown that ambient pH plays a major regulatory role in sclerotial development and suggest that a conserved pH-dependent signaling pathway is operative in *S. sclerotiorum*. Sclerotial development was favored by acidic ambient pH conditions but inhibited by neutral or alkaline pH conditions. To analyze whether *smk1* expression is linked with a pH signaling pathway, we determined *smk1* transcript abundance in cultures of *S. sclerotiorum* grown in media buffered with citrate-phosphate buffer at different pH values. The smk1 expression was lower at pH 7.0 (Fig. 4A) compared with that at acidic pH (pH 3.0). Therefore, we conclude that *smk1* expression correlates to pH.

A functional *pacC* homolog, *pac1,* previously was isolated in S. sclerotiorum (Rollins and Dickman 2001). Similar to that

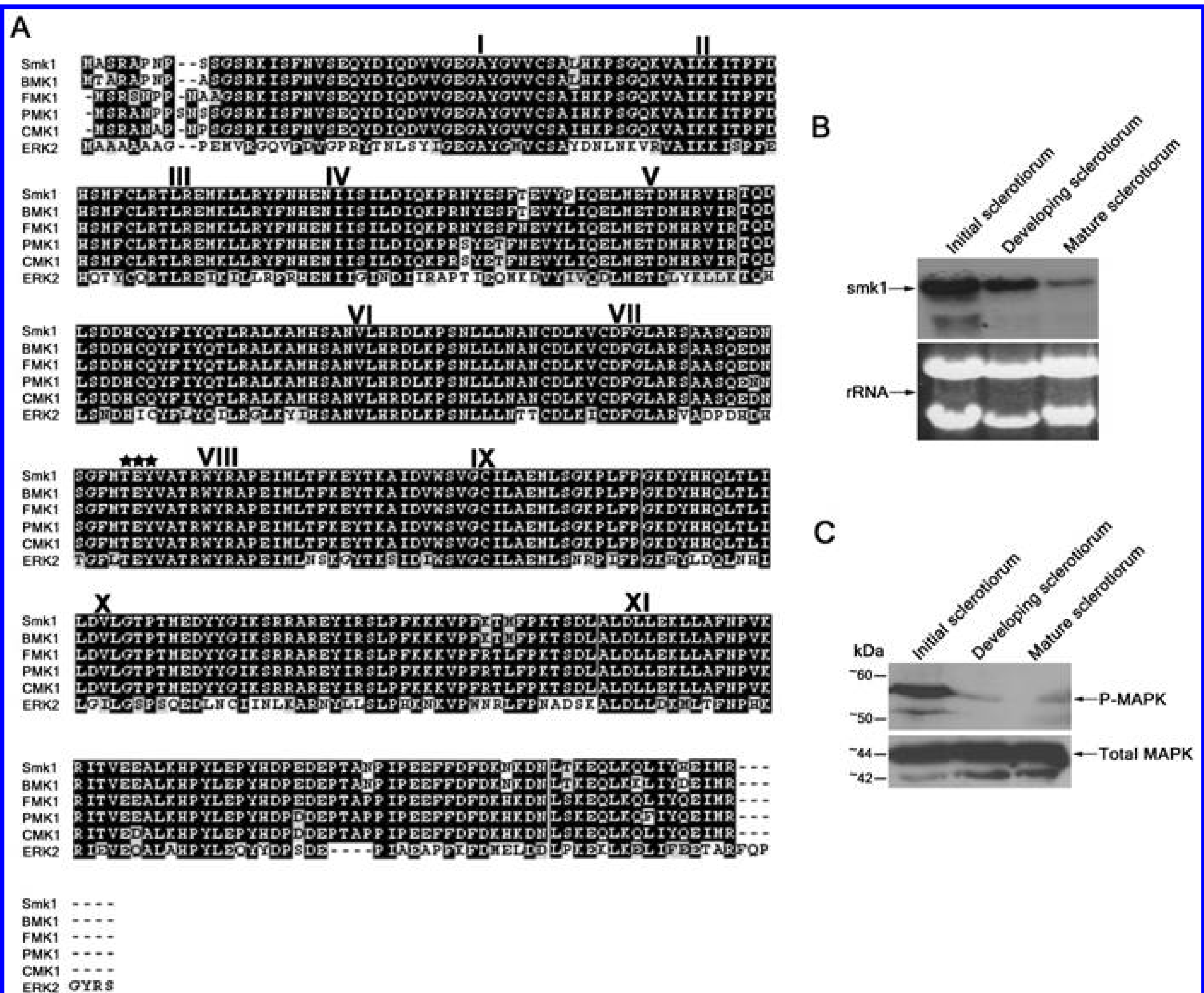

Fig. 2. Characterization of the *Sclerotinia sclerotiorum smk1* gene. **A,** Amino acid sequence alignment of the predicted *S. sclerotiorum smk1* gene product with other known mitogen-activated protein kinase (MAPK) proteins. Alignment with MAPKs from *Botryotinia fuckeliana* (BMK1), *Fusarium oxysporum* (FMK1), *Magnaporthe grisea* (PMK1), *Colletotrichum lagenarium* (CMK1), and *Mus muslucus* (ERK2) is shown. Identical amino acids are highlighted by the shaded background. Dashes indicate gaps in the alignment. The highly conserved MAPK phosphorylation sites (TEY) are noted with asterisks. Conserved kinase domains (I to XI) are marked. **B,** Developmental Northern-blot analysis of *smk1* expression. Total RNA (20 μg/lane) was isolated from sclerotia initials (3 to 4 days after inoculation), developing sclerotia (5 to 6 days after inoculation), and mature (melanized) sclerotia (7 to 8 days after inoculation). Northern blots were probed with *smk1* cDNA. For quantification, RNA was stained directly on the agarose gel with ethidium bromide. **C,** MAPK is activated during sclerotial initiation. Cultures from developmental stages described in B were harvested and cell lysates analyzed by Western analysis using antibodies that either specifically recognize the dually phosphorylated (active) forms of ERK1/2 (upper panel) or total ERK2 polypeptide (lower panel).

observed in the case of *A. nidulans pacC,* the accumulation of *pac1* transcripts paralleled increases in ambient pH. Our results showed that *smk1* transcript abundance correlates with a decrease in ambient pH. Thus, it is possible that a functional Smk1 signaling pathway may not affect *pac1* expression. To confirm this, we carried out Northern hybridization analysis to monitor *pac1* expression with total RNA from the *S. sclerotiorum smk1* antisense mutant, grown in minimal media at acidic and neutral pH levels with glucose or quinic acid as the carbon source. Consistent with the previous results showing the *pac1* expression pattern in *S. sclerotiorum* wild-type strain (Rollins and Dickman 2001), we found (Fig. 4B) that no matter whether *qa-2*-driven *smk1* antisense expression was induced by quinic acid or repressed by glucose, the *smk1* antisense mutant strain showed an equal intensity of the *pac1* hybridization signal under neutral pH conditions (pH 7.0) and almost no signal under low pH condition (pH 3.0). Based on these results, we conclude that expression of *pac1* under the various pH conditions tested does not require a functional *smk1*.

Exogenous OA induces *smk1* expression.

OA has been known to be a key pathogenicity determinant in *S. sclerotiorum* (Godoy et al. 1990) and ambient pH influences OA biosynthesis by regulating the enzymatic activity of oxaloacetate acetylhydrolase (Maxwell and Lumsden 1970; Rollins and Dickman 2001; Vega et al. 1970). Because *smk1* expression levels were dramatically higher under acidic conditions, we determined whether OA accumulation influences *smk1* expression. A time course experiment was conducted to analyze *smk1* expression following the addition of 12 mM OA to yeast-peptone/soluble starch (YPSS) media with an original neutral pH (pH 7.0). In order to determine whether the effect was specific to OA, 12 mM HCl or 12 mM potassium oxalate was added to the medium under the same conditions. Northern analysis showed that exogenous OA rapidly induced *smk1* expression (Fig. 5, upper panel). We also found that addition of HCl induced *smk1* expression in a comparable manner, but potassium oxalate did not (Fig. 5, middle and bottom panels). These results suggest that the decrease of ambient pH, rather than OA accumulation per se, is responsible for *smk1* expression.

Exogenous cAMP inhibits expression of *smk1* and MAPK phosphorylation.

It has been established previously that cAMP plays a key role in the early transition between mycelial growth and sclerotial development and that an increase in endogenous or exogenous cAMP levels inhibits sclerotial development in *S. sclerotiorum* (Rollins and Dickman 1998). Moreover, recent studies in a number of normal and transformed mammalian cell types

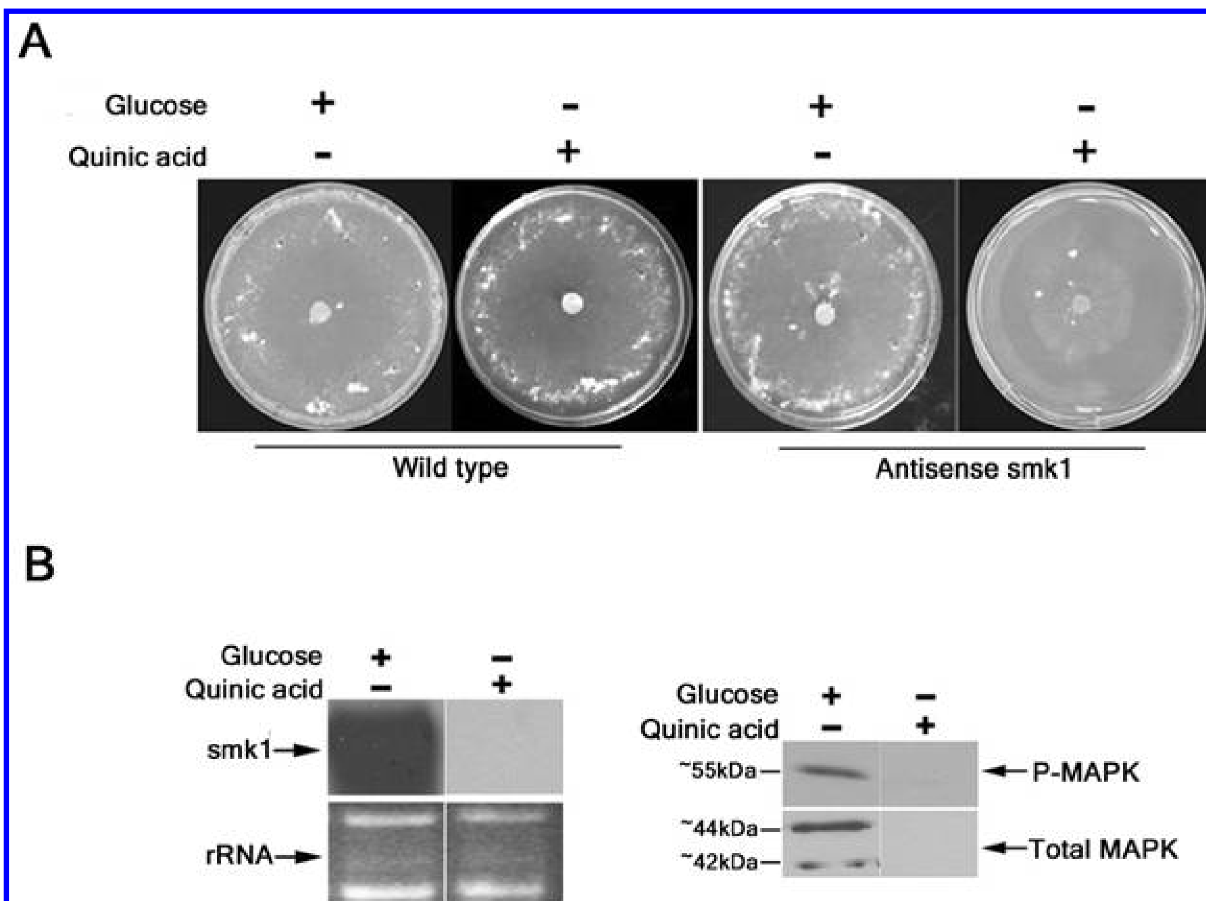

Fig. 3. Phenotype of a *Sclerotinia sclerotiorum smk1* antisense mutant. **A**, Wild-type isolate 1980 (left) and the *smk1* antisense mutant (right) were grown on petri dishes containing minimum media supplemented with 15 mM glucose (antisense expression repressed) or 15 mM quinic acid (antisense expression induced). The cultures were photographed 2 weeks after inoculation. **B**, Expression of *smk1* in the *S. sclerotiorum smk1* antisense mutant grown on induced (quinic acid added) or repressed (glucose added) media. For Northern blots analysis (left), total RNA (20 µg/lane) from cultures grown on minimal media amended with 15 mM glucose or 15 mM quinic acid was loaded and resolved on a formaldehyde agarose gel. Equal loading of RNA was confirmed by direct ethidium bromide staining. For Western blots analysis (right), sclerotial initials of the *smk1* antisense mutant were harvested from yeast-sucrose media after 4 to 6 days of incubation. After vigorous washing with sterile water, cultures were transferred to minimal media buffered at pH 3.0, and supplemented with 15 mM glucose or 15 mM quinic acid. Cell lysates were analyzed by Western blots using antibodies that specifically recognize either the dually phosphorylated (active) forms of ERK1/2 or total ERK2 polypeptide.

have shown that cAMP blocks the physiological actions of growth factors and these growth-inhibitory effects are via inhibition of the MAPK cascade (Cook and McCormick 1993; Graves et al. 1993; Indolfi et al. 1997; Schmitt and Stork 2001). Evidence for linkage between MAPK and cAMP pathways also has been shown recently in *U. maydis* (Lee and Kronstad 2002) and *Magnaporthe grisea*. (Xu and Hamer 1996). Thus, it is reasonable to speculate that cAMP-mediated inhibition of sclerotial development may involve changes in MAPK activity. Therefore, we examined the effect of cAMP on *smk1* expression and MAPK activation by Northern and Western analyses using cultures grown in media supplemented with 10 mM cAMP. Amending the growth medium with cAMP impaired sclerotial development (Fig. 6A). Consistently, we found that *smk1* transcription and MAPK phosphorylation also were suppressed by 10 mM cAMP (Fig. 6B).

Constitutive overexpression of *smk1* induces sclerotial development in the presence of high concentrations of cAMP.

Our results indicated that cAMP inhibits both *smk1* transcription and MAPK phosphorylation. In view of the effect of MAPK inhibition on sclerotial maturation, we reasoned that cAMP impairs sclerotial development via the inhibition of MAPK activation. Therefore, we investigated the effect of constitutive overexpression of *smk1* on sclerotial development. In this case, a transformant harboring a *qa-2*-driven *smk1* cDNA was used. Overexpression of *smk1* was verified by Northern and Western analyses (data not shown). Morphological analysis of the *smk1*-overexpressing strains showed that, under conditions repressing overexpression (glucose), cAMP (10 mM) inhibited sclerotial production in a manner similar to that of the wild type (Fig. 7, left panel). However, when *smk1* was overexpressed (Fig. 7, right panel), sclerotia were formed even in the presence of high concentrations of cAMP (10 mM). These findings suggest that constitutive overexpression of *smk1* bypasses the inhibitory effects of cAMP, resulting in the induction of sclerotial development, strongly supporting the notion that cAMP exerts its inhibitory effect on sclerotial development by suppressing MAPK activation.

DISCUSSION

Fungi possess varied specialized physiological and developmental strategies for dispersal, propagation, and long-term (season-to-season; year-to-year) survival. In *S. sclerotiorum*, these processes are mediated through the sclerotiorum, which can serve directly as a dispersal propagule in the disease proc-

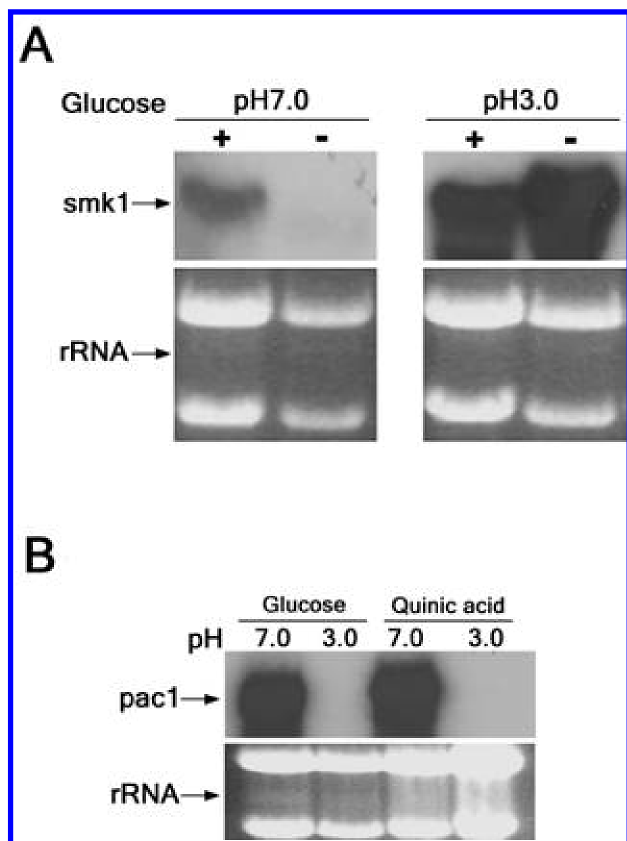

Fig. 4. A, *Sclerotinia sclerotiorum smk1* is transcriptionally regulated by ambient pH. RNA was extracted from mycelium harvested from 5-day-old cultures grown on yeast-sucrose (YPSS) media was transferred, for 24 h, to a minimal medium which was buffered at either pH 3.0 or 7.0, with or without glucose (0.3 g/ml) as the sole carbon source. **B,** Expression of *pac1* in the *smk1* antisense mutant. The mycelium was collected in the *smk1* antisense mutant following a 5-day incubation in YPSS media. After washing with sterile water, the culture was divided and transferred to a minimal medium, buffered at pH 3.0 or 7.0, and with glucose (15 mM) or quinic acid (15 mM) as the sole carbon source. Total RNA (30 µg/lane) was extracted from samples, fractionated on an agarose gel, blotted onto a nylon membrane, and hybridized with a radiolabeled *smk1* probe. Loading variations of different samples were normalized with direct ethidium bromide staining.

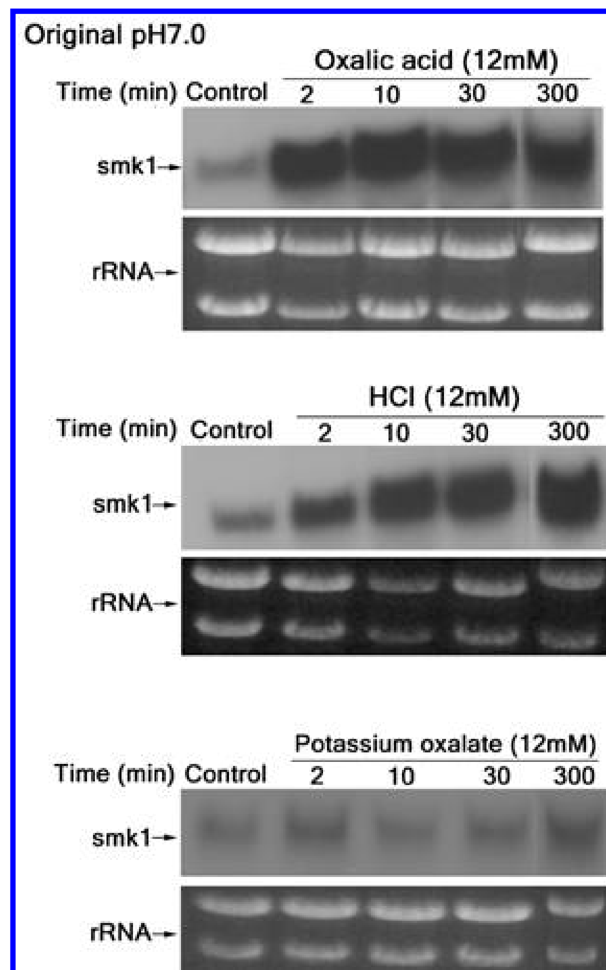

Fig. 5. Exogenous oxalic acid induces *smk1* expression at neutral pH. The fungus was grown for 5 days in yeast-sucrose (YPSS) media and the hyphae was transferred to fresh YPSS medium with an original pH of 7.0. Total RNA (30 µg/lane) was isolated after the incubation of the cultures with 12 mM oxalic acid (upper), 12 mM HCl (middle), or 12 mM potassium oxalate (bottom) for the indicated time periods. Blots were probed with labeled *smk1* cDNA. RNA was stained directly on the agarose gel with ethidium bromide to insure equivalent loadings.

ess. Thus, sclerotial development is fundamental to the disease cycle of this pathogen and understanding the regulation and relevant pathways of this differentiated structure may suggest novel strategies for controlling Sclerotinia diseases.

The MAPK family of proteins belongs to evolutionarily conserved signal transduction pathways mediating intracellular phosphorylation events involving cell growth, development, differentiation, and disease in response to extracellular stimuli (Cobb and Goldsmith 1995; Herskowitz 1995; Pelech and Sanghera 1992; Robinson and Cobb 1997). Using molecular and genetic approaches, we have demonstrated that the highly conserved MAPK homolog gene (*smk1*) from *S. sclerotiorum,* at least in part, is required for sclerotial development and likely interacts with pH- and cAMP-signaling pathways.

Smk1 functions in sclerotiorum development in *S. sclerotiorum*.

Smk1 encodes a polypeptide with subdomains characteristic of MAPK and is highly similar to many fungal MAPK polypeptides. This is the first instance that a MAPK has been shown to be specifically involved in signaling events linked with sclerotial development. Northern and Western blots showed that *smk1* transcription and activation are increased greatly during sclerotial initiation. We used both a pharmacological approach (PD98059, a specific inhibitor of the activation of MAPK by MAPK kinase) as well as a genetic one (*smk1* antisense expression) in order to determine the involvement of Smk1 in sclerotial development. In both cases, sclerotial maturation was severely impaired; therefore, we concluded that a functional Smk1 is required for regulating sclerotiorum development.

Smk1 functions in a pH-mediated signaling pathway in *S. sclerotiorum.*

Environmental pH has been shown to act as a key signal for growth, differentiation, and virulence in a variety of fungal pathogens (Caracuel et al. 2003; Davis et al. 2000; De Bernardis et al. 1998; Eshel et al. 2002; St Leger et al. 1998; Yakoby et al. 2000). A highly conserved pH-sensing signal transduction pathway whose terminal component is the zinc finger transcription regulator PacC/Rim101p has been identified and characterized in *A. nidulans* (Tilburn et al. 1995), *A. niger* (MacCabe et al. 1996); *Penicillium chrysogenum* (Suarez and Penalva 1996); *Yarrowia lipolytica* (Lambert et al. 1997); *Candida albicans* (Davis et al. 2000; El Barkani et al. 2000; Ramon et al. 1999), and *F. oxysporum* (Caracuel et al. 2003).

We found expression of *smk1* in *S. sclerotiorum* to be pH regulated, with transcript levels highest under acidic pH conditions (Figs. 4 and 5). Consistent with the role of MAPK activity in sclerotial development, this result suggests that Smk1 may regulate sclerotial development via a pH-dependent signaling pathway. Rollins and Dickman (2001) have shown previously that ambient pH is a regulatory cue for cellular events involved with *Sclerotinia* spp. pathogenicity and development. Sclerotial development was inhibited at a neutral or alkaline ambient pH where *smk1* expression also was inhibited, suggesting that sclerotial development involves pH-regulated MAPK activity. Moreover, ambient pH also regulates OA biosynthesis, which has been shown to be required for pathogenicity and sclerotial formation (Dickman and Mitra 1992; Godoy et al. 1990; Rollins and Dickman 1998, 2001). In this study, we observed a clear effect of medium acidification on the expression of *smk1*. Acidification of the extracellular milieu via OA induces an increase in *smk1* transcript abundance (Fig. 5A). The effect of OA likely is the result of environmental pH reduction because identical concentrations of HCl also induced *smk1* expression (Fig. 5B), whereas potassium oxalate amendments had no significant effect on *smk1* transcript levels (Fig. 5C). Together, these data provide compelling evidence that the reduction of ambient pH, which can be conferred by the accumulation of the *S. sclerotiorum*-produced OA, induces *smk1* expression. OA accumulation in *Sclerotinia* spp.-infected plant tissue has been noted for a number of years. Proposed functions of OA have included direct toxicity, chelation of Ca^{2+}, and vascular plugging as a result of oxalate crystals. Our results indicate that OA may be involved in the regulation of fungal gene expression and, in this case, associated with sclerotial development and, perhaps, virulence.

pH-mediated signaling has been found to be broadly conserved in the fungal kingdom (Penalva and Arst 2002) and the PacC gene product from *A. nidulans* is a key component in this signaling pathway, acting as a transcription factor to activate "alkaline" genes and repress "acidic" genes (Espeso and Arst 2000; Tilburn et al. 1995). Recently, Pac1, a functional homolog of PacC, was cloned and characterized in *S. sclerotiorum* (Rollins 2003; Rollins and Dickman 2001). However, it remains unclear if and how Pac1-dependent pH-mediated signaling regulates sclerotial development. The role of pH-dependent *smk1* expression in sclerotial development suggests that MAPK activity may influence Pac1 expression to regulate sclerotial morphogenesis in response to different environmental pH levels. To confirm this hypothesis, we investigated the *pac1* expression in the *smk1* antisense mutant background under acidic or neutral pH conditions. Based on our results,

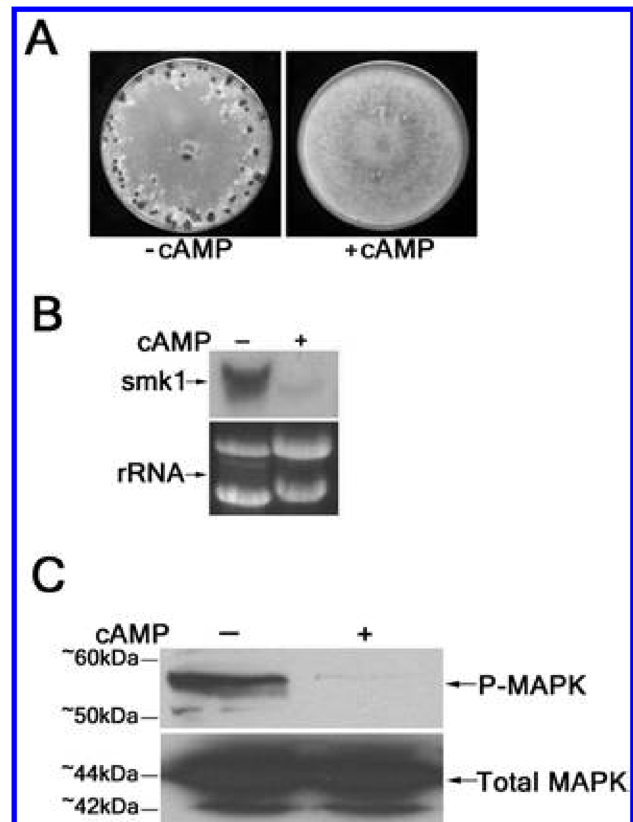

Fig. 6. Cyclic AMP (cAMP) inhibits *smk1* expression. **A**, Effect of cAMP on sclerotial development. A small agar-mycelium plug from a 5-day-old potato-dextrose agar (PDA) culture was inoculated to a fresh PDA media with or without 10 mM cAMP. The cultures were photographed after 14 days postinoculation. **B,** cAMP inhibits *smk1* transcription. The sclerotial initials were harvested from yeast-sucrose media supplemented with or without 10 mM cAMP and total RNA (20 µg/lane) was isolated and a Northern analysis was conducted by using *smk1* cDNA as probe. **C,** Cell lysates harvested from sclerotial initials treated with or without 10 mM cAMP were analyzed by Western blots using antibodies that specifically recognize either the dually phosphorylated (active) forms of ERK1/2 (upper panel) or total ERK2 polypeptide (lower panel).

we concluded that *pac1* expression was not dependent on a fully functional *smk1* (Fig. 4B). Consistently, Rollins and associates (*personal communication*) found that the transcript levels of *smk1* increased with decreasing ambient pH for both wild-type and pac1$^{-/-}$ deletion mutant strains. Taken together, our results suggest that an Smk1-dependent pathway may function coordinately with a Pac1-mediated signaling cascade to regulate the genes' expression during sclerotial development in response to different environmental pH conditions. In line with our findings, recent reports in *F. oxysporum* showed that a functional MAPK (Fmk1) signaling pathway did not influence *pacC* expression under different pH conditions, and PacC may cooperate with Fmk1 to regulate the expression of virulence determinants (Caracuel et al. 2003).

Smk1 functions in a cAMP-dependent signaling pathway in *S. sclerotiorum*.

cAMP, an important intracellular second messenger, is a central mediator for a number of biological activities in prokaryotic and eukaryotic cells (Kronstad 2000; Pastan and Perlman 1970; Robison and Sutherland 1971). In *M. grisea*, Xu and Hamer (1996) have demonstrated that cAMP signaling is linked with MAPK to regulate appressorial morphogenesis. In *S. sclerotiorum*, the effect of cAMP on sclerotial development has been studied and results revealed that increases in endogenous or exogenous cAMP levels specifically inhibit sclerotial development (Rollins and Dickman 1998). Our findings demonstrate that addition of cAMP inhibited *smk1* transcription, MAPK phosphorylation (Fig. 6B), and sclerotial development (Fig. 6A). Thus, it is conceivable that cAMP may inhibit sclerotial development by suppression of Smk1.

In a number of different types of cells, including fibroblasts, smooth muscle cells, and retinal pigmented epithelial cells, cAMP blocks cell growth and proliferation through the inhibition of ERK1/2-like MAPKs and this inhibition is primarily mediated by cAMP-dependent PKA (Bornfeldt and Krebs 1999; Ciullo et al. 2001; Cook and McCormick 1993; Graves et al. 1993; Howe and Juliano 2000; Indolfi et al. 1997; Sevetson et al. 1993). However, in preliminary experiments using specific cAMP-dependent PKA inhibitors, we found that, following pretreatment of the fungus with two PKA inhibitors (KT5720 and H89) at concentrations where the PKA activity was completely suppressed, the inhibition of Smk1 by cAMP was unaffected (*unpublished data*). These observations suggest that PKA does not participate in the molecular events linking cAMP to Smk1 in *S. sclerotiorum,* and a PKA-independent signaling may account for the inhibitory actions by cAMP. In the rice blast fungus, cAMP signaling has been established as being necessary for appressorium formation. However, even though cAMP commonly is associated with PKA, PKA deletion mutants still form appressoria, raising the possibility that a cAMP-dependent PKA-independent pathway was responsible and another effector of cAMP is involved. When a mutation in the regulatory subunit of PKA was made, appressorium formation was rescued in cAMP-defective mutants, thus indicating that divergent PKA catalytic subunits are required for appressorium development (Adachi and Hamer 1998). Such divergence also may be present in *N. crassa*, where two potential PKA catalytic subunits have been identified (Galagan et al. 2003), yet their roles in growth and development, as well as their substrate specificity, have yet to be defined. Recent studies involving follicle-stimulating hormone (FSH) showed repressed ERK1/2 in a cAMP-dependent but PKA-independent manner in primary cultures of Sertoli cells (Crepieux et al. 2002). cAMP also inhibits the proliferation of retinal pigmented epithelial cells through the inhibition of ERK1/2 in a PKA-independent manner (Hecquet et al. 2002), providing further support for PKA-independent cAMP signaling. Taken together, we cannot rule out the possibility that PKA plays an important role in sclerotiogenesis.

In conclusion, we have demonstrated that an *S. sclerotiorum* MAPK, Smk1, is a key regulator of sclerotial development via pH- and cAMP-sensing pathways. Future studies will aim to identify upstream and downstream components of Smk1-involved signaling pathways during pathogenic development and the relationship between cAMP and MAPK signaling pathways.

MATERIALS AND METHODS

Antibodies and reagents.

Monoclonal mouse anti-phosphorylated ERK antibodies (p-ERK(E-4)) and polyclonal rabbit anti-phospho p44/42 antibodies were purchased from either Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) or Cell Signaling Technology (Beverly, MA, U.S.A.), respectively. The inhibitor PD98059 was obtained from Calbiochem (Riverside, CA, U.S.A.).

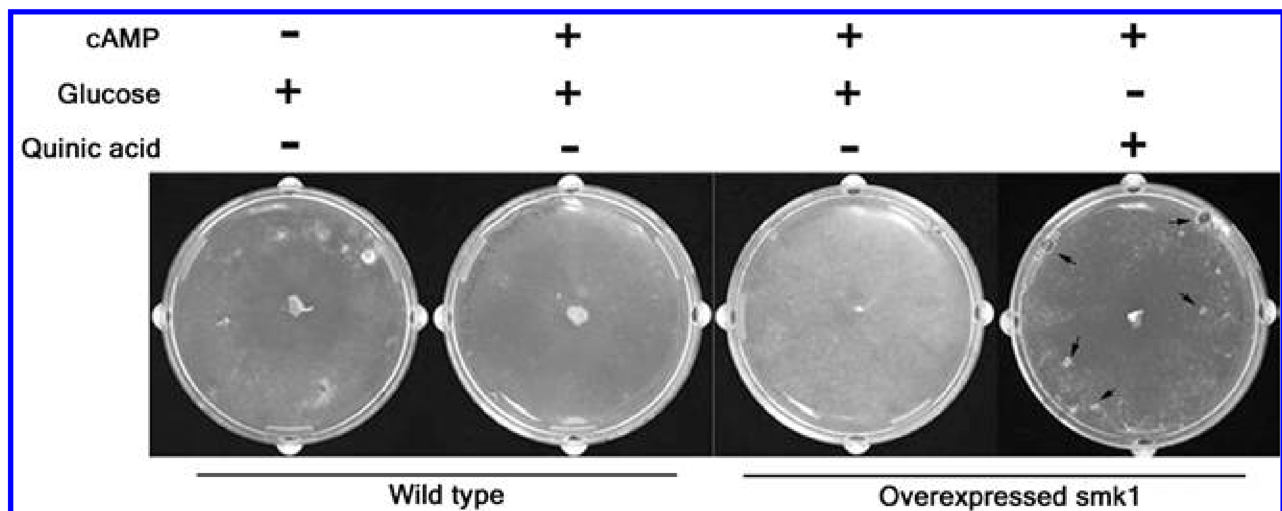

Fig. 7. Constitutive overexpression of *smk1* induces sclerotial development in the presence of high concentrations of cyclic AMP (cAMP). Wild-type isolate 1980 (left) and a constitutive overexpressing *smk1* mutant (right) were grown on plates containing minimal media supplemented with 15 mM glucose (repressed) or 15 mM quinic acid (induced), with or without 10 mM cAMP. Sclerotial development was monitored and photographed 2 weeks after inoculation. Arrows indicate the mature sclerotia.

cAMP, OA, and quinic acid (1,3,4,5-tetrahydroxycyclohexane-carboxylic acid) were purchased from Sigma (St. Louis). Potassium oxalate was obtained from ICN Biomedicals Inc. (Aurora, OH, U.S.A.). All other chemicals were from standard sources and were molecular biology grade or higher.

Fungal culture and growth conditions.

The wild-type strain of *S. sclerotiorum* "1980" used throughout this study (ATCC18683) was obtained from dry bean culls in western Nebraska (Godoy et al. 1990). This isolate was cultured routinely on PDA (Difco Laboratories, Detroit). Sclerotia initials, developing sclerotia, mature sclerotia, and hyphae were collected as follows: a single mycelium-agar plug was inoculated onto the center of PDA media in 9-cm-diameter petri dishes and grown at room temperature. Sclerotia initials normally were formed as fluffy white coalescing masses of hyphae after 2 to 3 days of growth; nonmelanized, developing sclerotia producing liquid exudates were observed after 4 days of growth; and melanized, mature sclerotia were available after 6 to 7 days of growth. To harvest hyphae, the medium surface was overlaid with cellophane before inoculation with the mycelial plug and cultures were grown for 3 days before sampling.

Isolation and manipulation of nucleic acids.

Standard techniques were employed in cloning and generation of recombinant plasmids (Sambrook et al. 1989). DNA was amplified by Pfu polymerase essentially as described by the manufacturer (Stratagene, La Jolla, CA, U.S.A.). DNA was sequenced by the dideoxy chain termination method (Sanger et al. 1977). For Northern blot analysis, cultures were harvested from either YPSS or minimal media after treatments as indicated and washed three times with sterile water. Total RNA was extracted in TriZOL reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Aliquots of RNA were loaded on formaldehyde denaturing gels in 1× MOPS buffer and transferred to a charged nylon membrane (Sambrook et al. 1989). RNA blots were hybridized in 7% sodium dodecyl sulfate (SDS), 0.5 M Na_2HPO_4 (pH 7.2), and 2 mM EDTA. The filters were hybridized at 65°C overnight and washed once for 10 min at room temperature (RT) with low-stringency washing solution (40 mM $NaHPO_4$, pH 7.2, 0.5% bovine serum albumen, 1 mM EDTA, 5% SDS), followed by 10 min at RT and two 10-min washes at 65°C with high-stringency washing solution (40 mM $NaHPO_4$, pH 7.2, 1 mM EDTA, 1% SDS). To ensure equal loading, samples were normalized by direct ethidium bromide staining of 17S rRNA (Yang and Dickman, 1999b).

pH studies.

To analyze the effect of ambient pH on *smk1* expression, 2-mm-diameter mycelium discs of isolate 1980, cut from the edge of a 4-day-old colony, were used to inoculate 500 ml of YPSS medium (4 g of yeast extract, 15 g of sucrose, 1 g of K_2HPO_4, and 0.5 g of $MgSO_4$ per liter) and cultured for 5 days with constant shaking. The entire culture was harvested by vacuum filtration and washed three times with sterilized water. Then, 10-ml aliquots of the washed mycelia were harvested by vacuum filtration and resuspended in 50 ml of fresh 0.1 M citrate-phosphate-buffered media (pH 3.0 and 7.0) with or without glucose (15 g/liter). These cultures were incubated for an additional 24 h prior to analysis.

Cloning an *S. sclerotiorum* MAPK.

We used a PCR-based approach to clone a *S. sclerotiorum* MAPK. Three degenerate primers, MK-1: GG(C/T)TT (A/C/G/T) A(A/C/G/T)(A/G)TC(A/C/G/T)C(G/T) (A/G)TG, MK-2: GT(A/C/G/T)GC(A/C/G/T)AT(A/G)AA(A/G)AA(A/G)AT, and MK-3: TC(A/C/G/T)GG(A/C/G/T)GC(A/C/G/T)C(G/T)(A/G)TA(A/C/G)(C/T)A, were designed according to the highly conserved amino acid motifs found in yeast MAPKs (FUS3, KSS1, and HOG1) and *M. grisea* MAPK (PMK1). First-strand cDNA was generated from polyadenylated mRNA prepared from a mycelial culture and used as template. The primary PCR reaction with primers MK-2 and MK-3 was conducted with the initial 5 cycles at a low annealing temperature (1 min, 94°C; 1 min, 45°C; and 1 min, 72°C), whereas the last 30 cycles were at a higher annealing temperature (1 min, 94°C; 1 min, 52°C; and 1 min, 72°C). Nested PCR was performed with primers MK-1 and MK-2 using the diluted primary PCR product (1 min, 94°C; 2 min, 52°C; and 2 min, 72°C; 30 cycles). The nested PCR products were cloned in a TOPO TA vector (Invitrogen) and sequenced. One of the PCR clones, showing high sequence similarity to known MAPK genes, was used to screen an *S. sclerotiorum* genomic library and an *S. sclerotiorum* cDNA library prepared with RNA from developing sclerotia (Rollins and Dickman 2001). The isolated MAPK-encoding gene was designated *Sclerotinia* MAPK 1 (smk1).

The nucleotide sequence of the *smk1* gene is available in the GenBank database under accession number AY351633.

Construction of plasmid vectors and fungal transformation.

An approximately 1.1-kb *Sma*I fragment containing the full-length *smk1* cDNA was amplified with primers MKSmaI-F (TCCCCCGGGATGGCAAGTCGT GCGCCTAA) and MKSmaI-R (TCCCCCGGGCTATCGCATGATCTCATG-GT) and cloned in the PCRbluntII TOPO vector (Invitrogen). The corresponding *Sma*I fragment was excised and ligated into pSO-1. pSO-1 was originated from pWFas51, a vector described by Fecke and associates (1993). pSO-1 carries the hygromycin phosphotransferase gene (*hph*) as a selectable marker replacing *am* (glutamate dehydrogenase) found in pWFas51. An antisense *smk1* construct was made by insertion of the inverted SMK1 cDNA into the *Sma*I site of pSO-1. Similarly, a constitutive overexpressing *smk1* construct was made by insertion of the in-frame *smk1* cDNA into the SmaI site of pSO-1. The orientation of both constructs was validated by sequence analysis. Expression of constitutively active *smk1* or antisense *smk1* was driven by the conditional *N. crassa* quinic acid (*qa-2*) promoter present in the pSO-1 plasmid. The constructs were used to transform protoplasts of *S. sclerotiorum* according to a standard polyethylene glycol-mediated transformation strategy (Rollins and Dickman 2001).

Western blotting.

Following different treatments, cultures of isolate 1980 were harvested and immediately frozen in liquid nitrogen, ground to a fine powder with a mortar and pestle, and suspended in extraction buffer (50% glycerol, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM dithiothreitol, 50 mM sodium fluoride, 0.2 mM phenylmethylsulfonyl fluoride). After 10 min of incubation on ice, samples were centrifuged for 10 min at 10,000 × *g* and the supernatant was transferred to a new tube. Cell lysate protein concentrations were quantified by the Bradford protein assay (Bradford 1976). For detection of phosphorylated and total ERK-type MAPKs, equal amounts of cell lysate proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%), blotted onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, U.S.A.), and probed with the corresponding antibodies according to the manufacturer's guidelines.

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