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Type 2A Phosphoprotein Phosphatase Is Required for Asexual Development and Pathogenesis of *Sclerotinia sclerotiorum*

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Sclerotinia sclerotiorum **is a necrotrophic, omnivorous plant pathogen with worldwide distribution. Sclerotia of *S. sclerotiorum* are pigmented, multihyphal structures that play a central role in the life and infection cycles of this pathogen. Plant infection depends on the formation of melanin-rich infection cushions, and secretion of hydrolytic enzymes and oxalic acid. Type 2A Ser/Thr phosphatases (PP2As) are involved in the regulation of a variety of cellular process. In the presence of cantharidin, a PP2A-specific inhibitor, hyphal elongation and sclerotia numbers were impaired whereas sclerotial size increased. We partially inactivated PP2A by antisense expression of the gene (*pph1*) encoding the PP2A catalytic subunit. When antisense expression was induced, almost complete cessation of fungal growth was observed, indicative of a crucial role for PP2A in fungal growth. RNAi-based gene silencing was employed to alter the expression of the 55-kDa R_2 (B regulatory subunit). Isolates in which *rgb1* RNA levels were decreased were slow growing, but viable. Melanin biosynthesis, infection-cushion production, and pathogenesis were significantly impaired in the *rgb1* mutants, yet theses mutants were pathogenic on wounded leaves. Reduced ERK (extracellular signal-regulated kinases)-like mitogen-activated protein kinase (MAPK) function conferred a reduction in NADPH oxidase and PP2A activity levels, suggesting a functional link between MAPK, reactive oxygen species, and PP2A activity in *S. sclerotiorum*.**

Sclerotinia sclerotiorum is a sclerotium-producing, phytopathogenic, filamentous ascomycete which is known to attack more than 400 plant species (Boland and Hall 1994; Bolton et al. 2006; Purdy 1979; Tu 1997). Sclerotia are hard, asexual, resting structures composed of vegetative hyphal cells which become interwoven and aggregate together (Willetts and Bullock 1992). Sclerotial development can be divided into three distinct stages: i) initiation—the appearance of small, distinct, initial forms of interwoven hyphae; ii) development—an increase in size; and iii) maturation—characterized by surface delimitation, internal consolidation, and pigmentation, and often associated with droplet secretion (Townsend and Willetts 1954).

Oxalic acid and lytic enzymes have been suggested to play significant roles in the pathogenesis of *S. sclerotiorum* on its hosts (Cotton et al. 2003; Favaron et al. 2004). In addition, infection of healthy tissue depends on the formation of complex melanin-rich structures called infection cushions which enable fungal penetration (Huang and Kokko 1992; Lumsden and Dow 1973). Ascomycota and the related Deuteromycota generally synthesize 1,8 dihydroxynaphthalene (DHN) melanin by oxidative polymerization of phenolic compounds via polyketide biosynthesis. In particular, DHN melanin has been shown to be essential for the rigidity of appressoria used to penetrate host plants by *Colletotrichum* and *Magnaporthe* spp. (Yamaguchi and Kubo 1992). Furthermore, melanin has been shown to be important for virulence in human pathogenic fungi, including *Cryptococcus neoformans* (Wang et al. 1995), *Aspergillus fumigatus* (Tsai et al. 1998), and *Wangiella dermatitidis* (Dixon et al. 1987).

In recent years, evidence has accumulated concerning the involvement of phosphorylative regulation in sclerotial development, and proteins such as mitogen-activated protein kinases (MAPKs) and cAMP-dependent protein kinase A (PKA) have been found to be part of this process (Chen et al. 2004; Harel et al. 2005; Rollins and Dickman 1998). The involvement of protein kinases in fungal development suggests that phosphatases, most likely, also are recruited to maintain balance in the phosphorylative regulation of sclerotial development. In fact, Harel and associates (2006) recently demonstrated that calcineurin, which is a Ser/Thr protein phosphatase (also known as PP2B), is involved in sclerotial development. Furthermore, a reduction in calcineurin activity also conferred reduced pathogenicity and impaired cell-wall biosynthesis in this fungus.

Type 2A phosphoprotein phosphatase (PP2A) is a major Ser/Thr phosphatase involved in several cellular signal-transduction pathways. The core structure of PP2A consists of a heterodimer (PP2AD) comprising a 36-kDa catalytic subunit (PP2Ac) and a 65-kDa anchoring A subunit. A third, variable, regulatory B subunit can associate with this core enzyme (Cohen 2003; Mayer-Yakel and Hemmings 1994; Van and Goris 2003). Based on their specific cellular and subcellular localization, the variable subunits can target the phosphatase to different tissues and cellular compartments. The presence of different regulatory subunits has been shown to determine the substrate specificity of the PP2A holoenzyme. These subunits also can modulate the response to agents that modify PP2A activity, altering the enzymes efficiency in dephosphorylating a specific substrate (Janssens and Goris 2001).

PP2As have been shown to play roles in the regulation of transformation, metabolism, transcription, RNA splicing, translation, differentiation, the cell cycle, oncogenic transformation, and signal transduction in a variety of organisms (Gallego and Virshup 2005; Lechward et al. 2001). Studies in *Saccharomyces*

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cerevisiae and *Schizosaccharomyces pombe* have demonstrated the involvement of PP2A in morphogenesis, actin distribution, and mitosis (Blacketer et al. 1993; Kinoshita et al. 1990; Lin and Arndt 1995; Tanabe et al. 2001; Van et al. 1992; Wang and Jiang 2003). In the filamentous fungus *Neurospora crassa,* PP2A has been shown to play an essential role in the regulation of hyphal growth (Yatzkan and Yarden 1995; Yatzkan et al. 1998). Inactivation of the B regulatory 55-kDa R_2 subunit (designated *rgb-1)* in *N. crassa* resulted in slow growth, morphological changes, female sterility, and overproduction of arthroconidia (Yatzkan and Yarden 1999). In addition, levels of the *N. crassa* circadian clock protein FREQUENCY in the *rgb-1* mutant were low, and resulted in a low-amplitude, long-period oscillation of the clock (Yang et al. 2004).

PP2A (also, to a certain extent, protein phosphatase type 1, whose catalytic subunits are structurally related) (Mumby and Walter 1993) is specifically inhibited by a variety of natural toxins (Holmes and Boland 1993). Among them are okadaic acid (which has been shown not to affect hyphal growth) (Yatzkan et al. 1998) and cantharidin, extracted from the blister beetle *Cantharia vesicatoria* (Li et al. 1993).

In all eukaryotes examined, reactive oxygen species (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-signaling 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 leading to differentiation states in microorganisms. In support of this idea, conidial differentiation in *Colletotrichum graminicola* has been shown to be accompanied by the activation of a manganese superoxide dismutase (SOD) (Fang et al. 2002). Another ROS-related enzyme, NADPH oxidase (NOX), is likely to serve as an endogenous source for ROS generation in the fungal cell. Deletion of *noxA* in *A. nidulans* was shown to block cleistothecium formation, but it did not affect vegetative growth or asexual development (Lara-Ortiz et al. 2003).

Some Ser/Thr phosphatases are known to be redox sensitive (Allen and Tresini 2000). Even though the nature of PP2A as an oxidant-sensitive phosphatase has yet to be elucidated, some studies support the notion that it is influenced by oxidative stress. For example, brain PP2A has been shown to be modified by a thiol-disulfide exchange to form an intermolecular disulfide bond (Foley and Kintner 2005). Phosphothreonine phosphatase activity of PP2A also has been shown to be inhibited in a dithiothreitol (DTT)-reversible manner by the cellular oxidants glutathione disulfide and hydrogen peroxide (Foley et al. 2004).

PP2A is a conserved enzyme and, based on the evolutionary proximity of *N. crassa* and *Sclerotinia sclerotiorum*, it is reasonable to postulate that this enzyme participates in the regulation of biochemical events affecting morphological changes in *S. sclerotiorum.* Nonetheless, the possible involvement of PP2A in sclerotial development or in plant pathogenesis has never been explored. We used the PP2A inhibitor cantharidin, antisense expression, and RNAi approaches to inhibit *pph1*, the *S. sclerotiorum* PP2Ac-encoding gene, and one of the B regulatory subunits, encoded by the *rgb1* gene. Inhibition of *pph1* resulted in aberrant hyphal morphology accompanied by growth arrest. Impaired *rgb1* expression, as obtained by RNAi-based gene silencing (demonstrated here for the first time in this organism), resulted in inhibition of sclerotial maturation and reduced pathogenesis. Based on our results, melanin biosynthesis is dependent on *rgb1* function. Moreover, we propose a functional link between MAPK and ROS signaling and PP2A activity in *S. sclerotiorum*.

RESULTS

Cantharidin alters the development of *S. sclerotiorum.*

To determine whether PP2A activity is involved in the development of *S. sclerotiorum*, we first examined the effect of the PP2A inhibitor cantharidin on hyphal growth and sclerotial formation. Quantitative analysis of the drug's effects demonstrated that i) hyphal elongation is negatively correlated with the concentration of cantharidin in the culture medium (50% effective concentration [EC_{50}] = 75 μM) (Fig. 1A), ii) the number of sclerotia formed is in reverse proportion to the concentration of cantharidin in the medium (EC_{50} = 68 μM) (Fig. 1B), and iii) the presence of 100 μM cantharidin in the fungal medium induces an increase of approximately 85% in average sclerotium size (Fig. 1C). These results demonstrate the inhibitory effect of cantharidin on *S. sclerotiorum* and support the possibility that PP2A is required for normal growth and development of this fungus.

pph1 is essential for *S. sclerotiorum* growth.

To further analyze the significance of PP2A in *S. sclerotiorum* growth, a genetic approach was used to complement the inhibi-

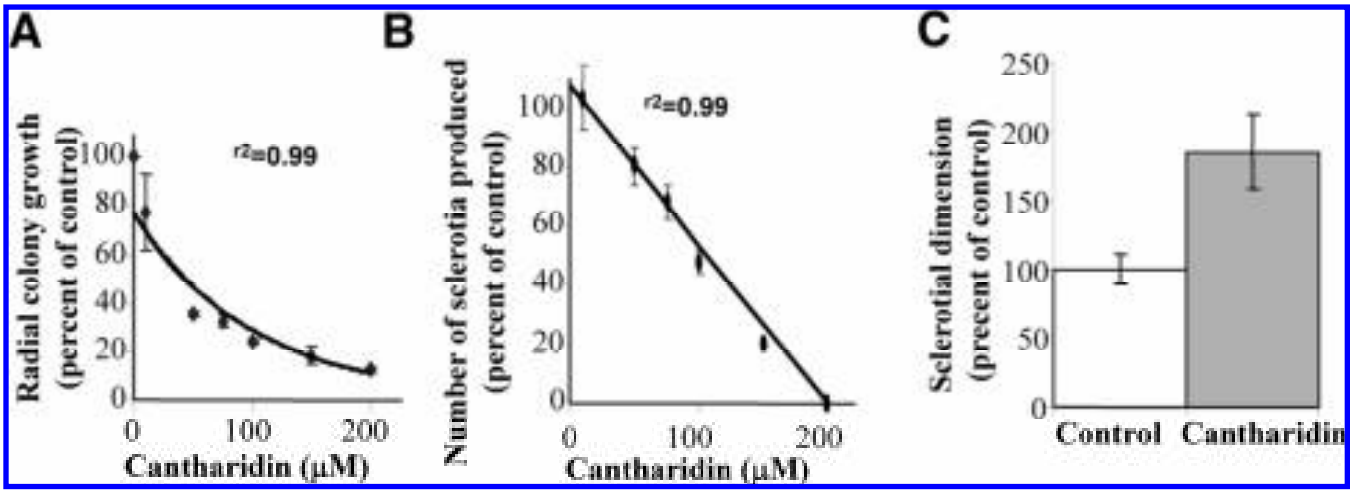

Fig. 1. Effect of cantharidin on colony growth and sclerotium formation in *Sclerotinia sclerotiorum*. **A,** Colony growth on solid media supplemented with different concentrations of cantharidin. **B,** Effect of cantharidin on sclerotium formation tested under conditions favoring nearly synchronized sclerotial formation. **C,** Effect of cantharidin on sclerotial size (width by length [cm^2]/control). Bars indicate standard error.

tor-based experiments. We first cloned a fragment of the *S. sclerotiorum pph1* gene (discussed below). This gene contains seven exons and encodes a predicted polypeptide of 267 amino acids, which is highly similar to PP2A proteins in other organisms. An approximately 850-bp DNA fragment of *pph1* was inserted into the pSO-1 vector in antisense orientation, downstream of the *N. crassa* quinic acid 2 gene promoter (Fig. 2A). The resulting construct, pAE3, was used to transform the wild-type strain of *S. sclerotiorum*. Three independent *pph1* mutants (as verified by polymerase chain reaction [PCR]) (data not shown) were isolated and all exhibited an identical phenotype. Specifically, after being transferred to quinic acid-containing medium, newly formed hyphae barely protruded from the mycelial disk used for inoculation and, shortly thereafter, hyphal growth ceased altogether. One of the mutants (designated PAS1) was chosen for further analyses. As hyphal growth was arrested in the presence of quinic acid and as we, nonetheless, wanted to determine if the induced antisense expression reduced pph1 transcript levels, we first cultured both wild type and PAS1 on a sterile cellophane membrane layer placed over the potato dextrose agar (PDA) medium. After the fungus had grown over most of the petri dish, the culture was transferred, by removing the cellophane membrane, and placed on either quinic acid (15 mM) or glucose (15 mM) medium for 16 h, prior to nucleic acid and protein extraction. When the expression of *pph1* in antisense orientation was induced by quinic acid, *pph1* transcript levels were markedly reduced in the PAS1 antisense transformant (Fig. 2B).

Total PP2A phosphatase activity was measured in the PAS1 strain grown on antisense induced or repressed media. The PAS1 and wild-type strains were grown first on PDA and then transferred to a medium containing quinic acid (15 mM) or glucose (15 mM). There were no significant differences between PP2A activity levels in extracts of the PAS1 versus wild-type strains when grown in the presence of glucose. Interestingly, even though a slight increase in radial growth rate of the wild type was observed in the presence of quinic acid, this was accompanied by a mild reduction (11 ± 3%) in PP2A activity (Fig. 2C). In sharp contrast, a 70 ± 11% reduction in relative PP2A activity was measured in the extracts of the PAS1 strain grown in the presence of quinic acid (Fig. 2C). These results confirmed our hypothesis concerning PP2Ac's essential role in *S. sclerotiorum* growth and development.

rgb1 is required for sclerotial development in *S. sclerotiorum*.

Because *pph1* was shown to be an essential gene for *S. sclerotiorum* (as is the case for *pph-1* in *N. crassa*) (Yatzkan and Yarden 1995) and PP2Ac is a key component that is common to all PP2As, we assumed that examining the expression of one of the variable subunits during *S. sclerotiorum* development may provide additional information concerning PP2A's putative role in *S. sclerotiorum* development. We first used a real-time reverse-transcriptase (RT)-PCR approach to determine expression levels of the 55-kDa R_2 B regulatory subunit (des-

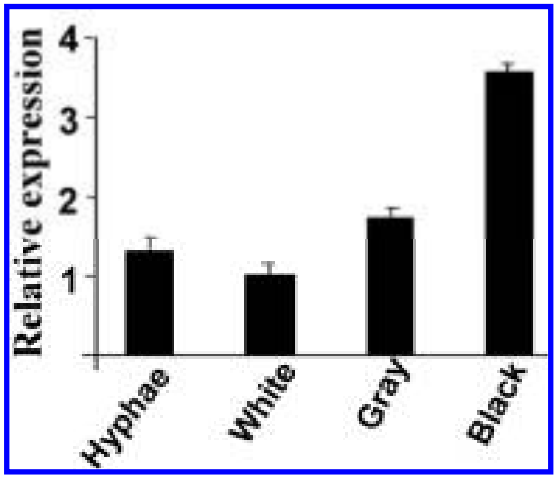

Fig. 3. Changes in *rgb1* expression during sclerotial development as determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). The quantity of *rgb1* cDNA measured by RT-PCR was normalized to that of β-tubulin cDNA in extracts from each developmental phase. Gray sclerotia are those that are just starting to accumulate melanin. Data shown are the average expression levels based on two experiments (originating from individual biologically replicated cultures with independent RNA extractions). Bars indicate standard error. The abundance of cDNA from white sclerotia samples was arbitrarily assigned a value of 1.

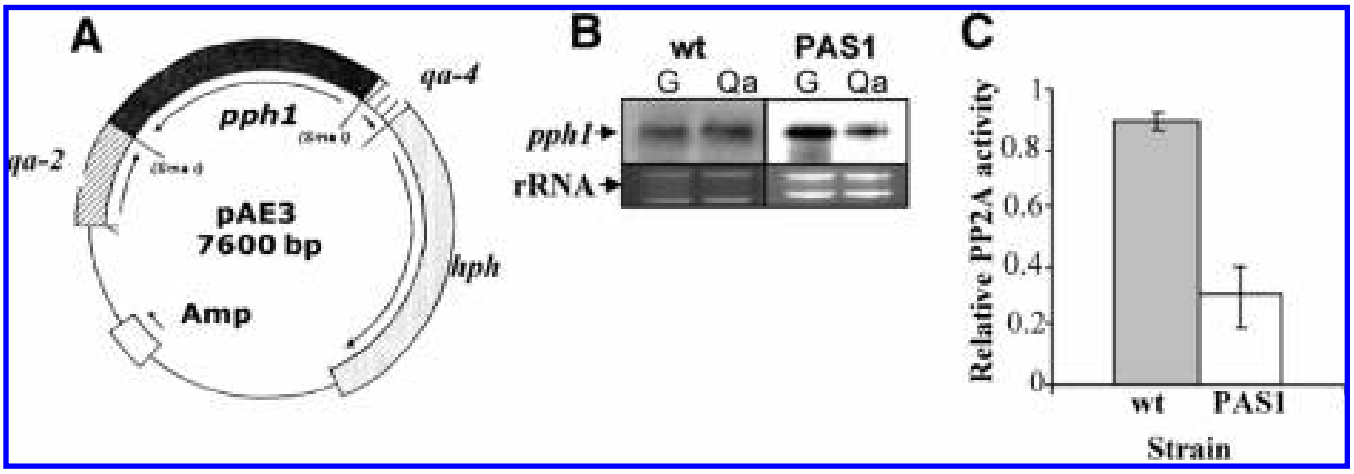

Fig. 2. Construction of a *pph1* antisense RNA expression vector (pAE3) and functional analysis of the PP2A antisense strain (PAS1). **A,** A 1-kbp fragment of *pph1* was inserted in antisense orientation between the promoter region of the *Neurospora crassa qa-2* gene and a 380-bp fragment which includes regions containing polyadenylation and termination signals from the *N. crassa qa-4* gene of pSO-1. **B,** Expression of *pph1* in the *Sclerotinia sclerotiorum* PAS1 strain grown on *qa-2*-induced (Qa; 15 mM quinic acid) or repressed (G; 15 mM glucose) medium as determined by Northern blot analysis; wt = wild type. Equal loading of RNA was confirmed by direct ethidium-bromide staining (lower panel). **C,** Relative specific activities of PP2A (ratio of antisense-induced over antisense-repressed) in extracts of the *S. sclerotiorum pph1* antisense transformant (PAS1) and the wild-type strain. PP2A activity was determined by measuring the dephosphorylation rate of the RII synthetic phosphopeptide substrate. Data presented is the average of three independent experiments. Error bars indicate variance (*t* test, $P < 0.05$).

ignated *rgb1*, which contains six exons and encodes a conserved polypeptide of 484 amino acids) during different morphological stages of asexual development of *S. sclerotiorum.* Differences in *rgb1* expression were observed during the different phases of sclerotial development. When compared with the expression levels detected in white sclerotia (which was comparable with that measured in hyphae), an almost 2-fold increase in *rgb1* expression level was observed in gray sclerotia (a stage preceding completion of sclerotial melanization) and a 3.5-fold increase in transcript level was measured in mature black sclerotia (Fig. 3).

To elucidate the possible roles *rgb1* may play in sclerotial development, we produced a strain in which *rgb1* expression levels were reduced via an RNAi *rgb1* cassette. After cloning the *S. sclerotiorum rgb1* gene (discussed below), two *rgb1* DNA fragments of approximately 1.1 kbp in size were inserted into the pSilent vector in opposing orientation, downstream of the *trp*C promoter. The resulting construct, pAE21 (Fig. 4A), was used to transform the wild-type strain of *S. sclerotiorum*, and approximately 20 pAE21-containing transformants (verified by PCR; data not shown) were isolated. Northern blot analysis was used to assess the abundance of *rgb1* transcript level in the different strains (Fig. 4B). Among the transformant strains, 3D and 8D exhibited markedly reduced expression levels of *rgb1* and were chosen for further analysis. The transformants exhibited significantly slower hyphal elongation rates (6 ± 1% of the wild type) as determined by measuring colony area after 3 days, yet hyphal morphology (including hyphal branching patterns) was indistinguishable from that of the wild type. In addition, in the *rgb1* mutants, sclerotial maturation was severely impaired (Fig. 4C) and the mutants were unable to accumulate melanin, even weeks after the colony covered the plates. Thus, the near absence of *rgb1* transcript does not result in fungal lethality (as is the case with *pph1*) but affects radial growth and severely impairs the process of sclerotium development.

The *rgb1* mutants of *S. sclerotiorum* exhibit impaired pathogenesis.

To date, the possible involvement of PP2A in fungal pathogenesis has not been analyzed. To determine whether a reduction in *rgb1* expression would confer a change in the pathogenic capability of *S. sclerotiorum* (even though hyphal morphology apparently was unaffected), we inoculated detached tomato leaves with agar plugs colonized with the *rgb1* mutants (3D or

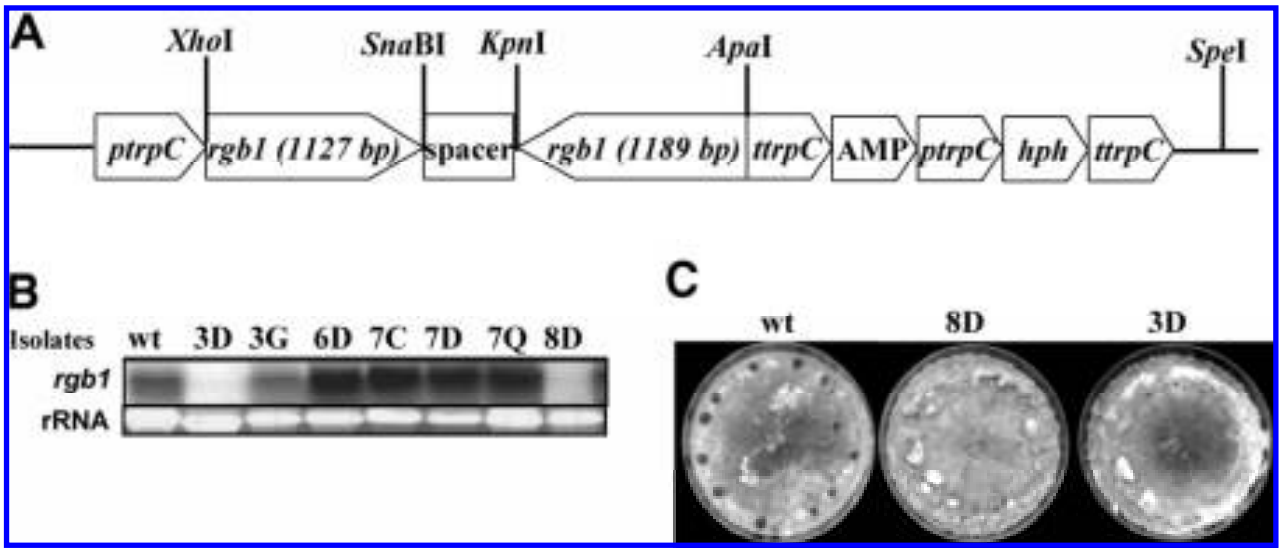

Fig. 4. Construction of an *rgb1* RNAi expression vector (pAE21) and functional analysis of *rgb1* mutant strains. **A,** A 1.1-kbp fragment of *rgb1* was inserted in sense orientation between an *Aspergillus nidulan*s trpC promoter and a spacer DNA and another 1.1-kbp fragment of *rgb1* was inserted in antisense orientation between this DNA spacer and the trpC terminator. **B,** Expression of *rgb1* in isolates containing the pAE21 construct as determined by Northern blot analysis. Equal loading of RNA was confirmed by direct ethidium-bromide staining (lower panel). **C,** Phenotype of the *rgb1*-silenced transformants 3D and 8D (right) versus wild type (wt) (left).

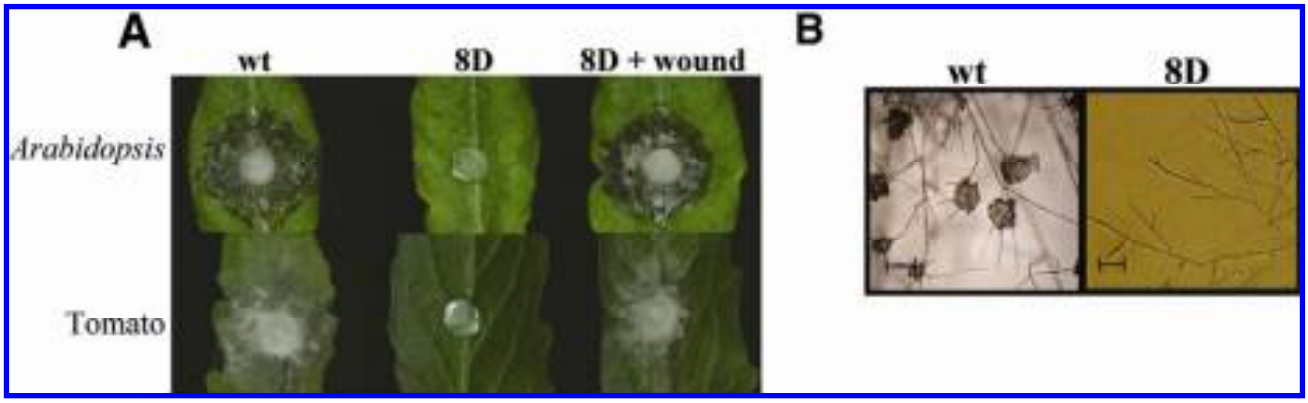

Fig. 5. Effects of *rgb1* inhibition on pathogenicity and infection-cushion formation. **A,** Tomato (cv. Bonny Best) or *Arabidopsis thaliana* (accession Co-0) leaves were inoculated with water-agar plugs precolonized with the wild type (wt) or an *rgb1* RNAi transformant (8D). Symptom development on wounded leaves inoculated with 8D is shown on the right panel (8D+wound). **B,** Effect of RNAi-based inhibition of *rgb1* on infection-cushion formation. Potato dextrose agar plugs colonized with the wild type (wt; left) or an *rgb1* RNAi transformant (8D; right) 5 days after transfer to a hydrophobic surface. Bar = 100 μM.

8D) or with the wild-type control. When compared with the fully pathogenic wild type, pathogenicity of the *rgb1* mutants was totally abolished and the mutants were unable to produce disease symptoms (as determined by monitoring the number of leaves in which necrotic lesions appeared) (Fig. 5A) even 5 days postinoculation. Because *S. sclerotiorum* is a broad host range pathogen, we expanded the pathogenicity assay to an additional host: *Arabidopsis thaliana.* In this case as well, all leaves inoculated with the wild type exhibited disease symptoms. Inhibition of *rgb1* expression levels conferred a total lack of fungal virulence and the mutants failed to infect the cruciferous plants as measured 5 days postinoculation (Fig. 5A), indicating that this phenomenon is not restricted to the interaction between *S. sclerotiorum* and tomato.

Studies of the infection process of *S. sclerotiorum* on bean hypocotyls and pea pods have documented the formation of infection-cushion structures which may participate in the penetration and pathogenicity process (Huang and Kokko 1992; Lumsden and Dow 1973). To determine whether a reduction in *rgb1* expression confers a change in the production of infection cushions, we placed agar plugs colonized with the *rgb1* or wild-type strains on a transparent hydrophobic surface (empty petri dishes) (Harel et al. 2006). The wild type developed typical infection cushions after 24 h. However, infection cushions were not observed in the *rgb1* mutants 3D and 8D (Fig. 5B), even after 5 days, suggesting that, in addition to sclerotial production, this developmental process also is impaired in the *rgb1* mutants.

In order to determine whether the lack of infection cushion development significantly contributed to the loss of pathogenicity, we repeated the leaf inoculation experiments, yet wounded the leaves prior to inoculation. When inoculated with either of the *rgb1* mutants, the wounded plant leaves of both tomato and *Arabidopsis* exhibited disease symptoms similar to those observed in the wild-type control (Fig. 5A), suggesting that infection cushion formation may be an essential component of the disease-causing process.

Infection cushions and sclerotia are melanin-rich structures and the production of both is impaired in the *rgb1* strain. Therefore, we sought to determine whether the defect in *rgb1* affects the melanin biosynthesis pathway. While screening for genes related to melanin biosynthesis (on the basis of sequence similarity), the putative genes of the DHN melanin biosynthesis pathway, which is common to many filamentous fungi (Henson et al. 1999), were identified in the *S. sclerotiorum* genome. The precursor of the DHN melanin biosynthesis pathway is acetate. Therefore, we added sodium acetate to the medium to determine whether the deficiency in melanin was due to lack of this precursor. When 2.5 mM acetate was added to the medium, the radial growth rate of the *rgb1* mutants increased by approximately 50% (to 22 ± 2% of the wild type) (Fig. 6), suggesting some compensatory effect of the amendment. However, even though improved hyphal growth was evident, the addition of acetate was not sufficient to complement the phenotypic defects in melanin biosynthesis, sclerotial maturation, or infection-cushion production, suggesting that PP2A also may regulate another downstream enzyme or enzymes in the DHN pathway. We also concluded that the positive effect of growth was not a result of acetate-induced pH reduction because addition of oxalic acid (acidifying the medium to pH 4.5, as conferred by the acetate amendment) had no such effect.

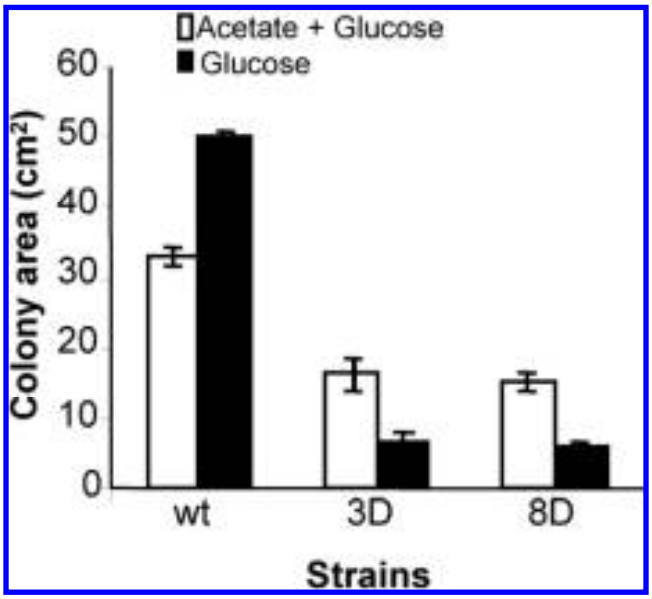

Fig. 6. Colony area (cm^2) of isolates 3D, 8D, and the wild type (wt) with or without 2.5 mM sodium acetate, after 3 days of growth. Data shown is based on the average of three experiments. Bars indicate standard error.

Part of the production of acetate from oxaloacetate in *S. sclerotiorum* is accompanied by production of the pathogenicity factor oxalic acid (catalyzed by the enzyme oxaloacetate acetylhydrolase) (Rollins and Dickman 2001; Sexton et al. 2006). Thus, because the acetate amendment improved radial growth of the fungus, we analyzed the production of oxalic acid in the *rgb1* mutants. Cultures were harvested after hyphal growth reached a diameter of 8 cm and the accumulation of oxalic acid in the medium was measured. We found that secreted levels of oxalic acid in the *rgb1* mutants were highly similar to those secreted by the wild-type control (0.15 to 0.3 mg/liter/day). Thus, reduced *rgb1* expression had no significant effect on the production of this factor, which is considered a key virulence determinant in *S. sclerotiorum* (Burke and Rieseberg 2003; Cessna et al. 2000; Donaldson et al. 2001; Godoy et al. 1990; Guimaraes and Stotz 2004).

The MAPK pathway regulates PP2A activity via NADPH oxidase.

Recent studies support the notion that PP2A is influenced by oxidative stress (Foley and Kintner 2005; Foley et al. 2004). Therefore, we examined whether inhibiting the superoxide producer NOX affects PP2A activity. Extracts from wild-type hyphae that had grown on PDA in the presence or absence of 50 μM NOX inhibitor diphenyleneiodonium chloride (DPI) were assessed for PP2A activity. In three independent experiments, we measured a 30 ± 10% reduction in PP2A activity in extracts from cultures grown in the presence of the NOX inhibitor relative to the control (Fig. 7A). As expected, DPI had no observable effect on the activity of another ROS-related enzyme, SOD. Nox1 activity and expression have been shown to be activated by the MAPK pathway in mammalian cells (Mitsushita et al. 2004), and an ERK (extracellular signal-regulated kinases)-type MAPK, Smk1, has been shown to be required for sclerotial development in *S. sclerotiorum* (Chen et al. 2004). Therefore, we examined NOX activity in extracts from hyphae of the *smk1* mutant grown in the presence of either quinic acid or glucose (inducing or repressing *smk1* expression, respectively). A significant reduction in NOX activity was observed in extracts from the *smk1* strain grown in the presence of quinic acid (as detected by an in-gel assay), whereas the activity of SOD (monitored as a control) was not altered (Fig. 7B). We postulated that Smk1 positively regulates NOX activity which, in turn, regulates PP2A activity; therefore, we further examined whether a link could be established between Smk1 and PP2A's involvement in sclerotial maturation. PP2A activity was measured in extracts from hyphae of the *smk1*

mutant grown in the presence of either quinic acid or glucose. A 40 ± 7% reduction in relative PP2A activity was measured in extracts of the *smk1* strain grown in the presence of quinic acid, whereas addition of quinic acid to a wild-type culture resulted in only a mild reduction in PP2A activity (11 ± 3%), indicating that smk1 is required for normal PP2A activity levels (Fig. 7C). Thus, based on these results, we concluded that, even though it is highly likely that different cellular components can affect PP2A, the activity of this enzyme is dependent on the function of both Smk1 and NOX.

DISCUSSION

S. sclerotiorum is an omnivorous plant pathogen with a broad host range and worldwide distribution. Understanding the sensing and regulation processes that determine the transition from one developmental stage to the next and, particularly, sclerotial development and germination, can prove advantageous in the formulation of new strategies for controlling Sclerotinia diseases. In recent years, evidence has accumulated concerning the involvement of phosphorylative regulation in sclerotial development and pathogenesis. The use of phosphatase inhibitors, which are able to penetrate living cells, has been a breakthrough in the study of the functions of protein phosphatases in vivo. Li and Casida (1992) isolated a cantharidin-binding protein from mouse liver cytosol which was identified as PP2A and, since then, cantharidin has been shown to be a potent inhibitor of PP2A in vivo and in vitro (Li et al. 1993; Yatzkan et al. 1998). Okadaic acid also has been shown to be a potent inhibitor of cantharidin (which is structurally unrelated to okadaic acid), binding to this protein. Despite the fact that okadaic acid is one of the most specific inhibitors of PP2A known, this drug does not inhibit hyphal growth of *N. crassa* (Yatzkan et al. 1998); therefore, we used cantharidin in this study. Our results indicated that cantharidin inhibits hyphal elongation in a concentration-dependent manner with an EC_{50} = 75 μM, which is approximately twofold more toxic than the drug's effect on *N. crassa* (Yatzkan and Yarden 1995). We also showed that, although cantharidin decreases the number of sclerotia produced in a concentration-dependent manner, sclerotial size increases by approximately 85% in the presence of 100 μM (which is close to the EC_{50} of hyphal elongation) of this drug. This phenomenon (i.e., the production of fewer but more massive sclerotia) has been described by Chet and Henis (1975), who explained that this indicated that the "trigger" processes inducing initiation of sclerotium formation are different from those involved in the further development and maturation of these structures. In addition, as yet to be identified environmental or food base conditions (including spent medium) have been shown to affect sclerotial size in *S. sclerotiorum* (Bedi 1958).

To analyze the role of the gene encoding the PP2A catalytic subunit, *pph1*, in *S. sclerotiorum* growth and development, we constructed the PAS1 mutant. Because we assumed that *pph1* might be an essential gene (as it is in *N. crassa*), we used an inducible *pph1* antisense construct which enabled us to control the antisense expression. When antisense expression was induced by quinic acid, inhibition of fungal growth was observed, confirming our hypothesis that PP2A plays a crucial role in fungal growth. Based on these results, along with the observed effect of cantharidin on hyphal growth, PP2A may serve as a potential target for antifungal intervention. In fact, the herbicidal activity of endothal (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid), which is structurally related to cantharidin, may be associated with inhibition of PP2A (Li and Casida 1992; Li et al. 1993). Furthermore, because protein phosphatases represent a major and largely untapped therapeutic target, researchers have strived to address this potential target in the development of novel small-molecule therapeutic protein phosphatase inhibitors, well beyond the field of plant protection (Sakoff and McCluskey 2004).

PP2Ac was found to be essential for fungal growth and it was not possible to determine its specific involvement in morphogenesis and pathogenesis; therefore, we examined the expression of one of the PP2A variable subunits during *S. sclerotiorum* development. The observed increase in *rgb1* transcript level during the process of sclerotium maturation implied that *rgb1* may, in fact, be involved in the maturation process. Dormant sclerotia of *Physarum polycephalum* have been shown to store mRNA sequences in association with a distinct set of proteins (Adams et al. 1981); therefore, it is possible that *rgb1* transcript accumulation in the sclerotia is a mechanism by which the sclerotium prepares itself for its dormant phase or the germination process.

To further support the importance of *rgb1* in the sclerotium-formation process, we produced a strain in which *rgb1* expression levels were reduced via an RNAi *rgb1* cassette. The use of RNAi for exploring gene function has been demonstrated in several species of *Aspergillus* (McDonald et al. 2005; Mouyna et al. 2004), *Magnaporthe grisea* and *Colletotrichum lagenarium* (Nakayashiki et al. 2005), *Cryptococcus neoformans* (Liu et al. 2002), *Fusarium graminearum* (McDonald et al. 2005), *N. crassa* (Goldoni et al. 2004), *Venturia inequalis* (Fitzgerald et al. 2004), and *Ophiostoma* spp. (Tanguay et al. 2006). This

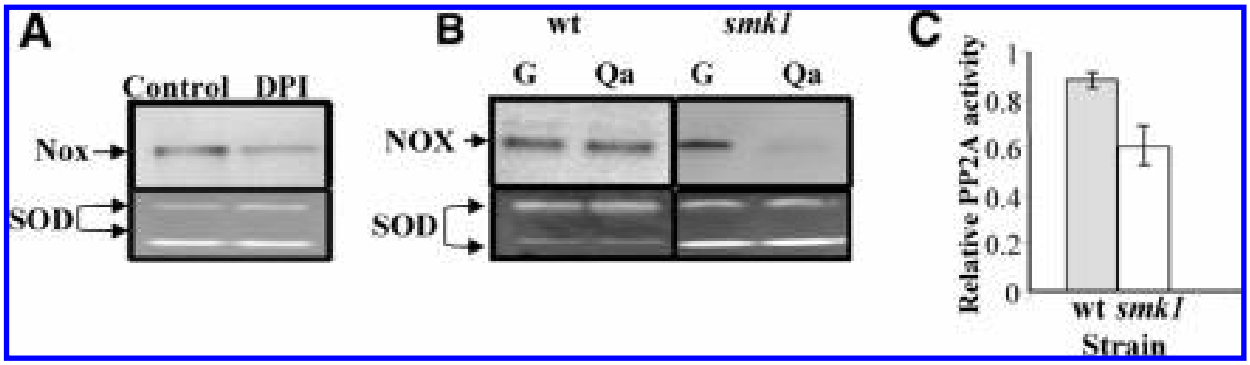

Fig. 7. A, NADPH oxidase (NOX) and superoxide dismutase (SOD) activity in a 10% polyacrylamide gel as detected in the presence or absence of the NOX inhibitor diphenyleneiodonium chloride. Typical in-gel NOX and SOD activity (one of three experiments performed with extracts from independent cultures) is shown. **B,** NOX activity in the inducible antisense *smk1* mutant in the presence of quinic acid (Qa) or glucose (G). SOD activity served as control (bottom panel). All activity assays were performed in a 10% polyacrylamide gel; wt = wild type. Typical in-gel NOX and SOD activity (one of three experiments performed with extracts from independent cultures) is shown. **C,** Relative specific activities of PP2A (ratio of antisense-induced over non-antisense-repressed) in extracts of the *Sclerotinia sclerotiorum smk1* antisense transformants or the wild-type (wt) strain. Data presented is the average of three independent experiments. Error bars indicate variance (*t* test, $P < 0.05$).

is the first study to show that RNAi constructs can be used to reduce gene expression in *S. sclerotiorum*. Unexpectedly, some of the obtained transformants exhibited increased expression levels of *rgb1* (Fig. 4B). One explanation for this may be chromosomal integration of only part of the cassette, resulting in the involvement of an additional, expressed copy of *rgb1* that lacks the corresponding hairpin arm. Among the *rgb1* RNAi construct transformants, strains 3D and 8D exhibited markedly reduced expression levels of *rgb1*, as verified by Northern blot analysis. When grown on PDA medium, these mutants grew slowly and produced white sclerotia, apparently as a result of inhibited melanin production, which further supports our hypothesis of *rgb1*'s involvement in sclerotial development and perhaps in melanin biosynthesis.

Although PP2As have been shown to play roles in a variety of cellular and developmental processes, their involvement in a pathogenetic process (be it animal or plant) has never been demonstrated. *S. sclerotiorum* pathogenicity assays revealed that the *rgb1* mutants are nonpathogenic on detached leaves of *A. thaliana* or tomato. Because wounding of the leaves prior to inoculation with the *rgb1* mutants resulted in wild-type levels of symptom development, it is highly possible that the *rgb1* mutants' inability to produce infection cushions contributed to their impaired pathogenic capacity (in addition to the fact that no mature sclerotia were produced). It has been shown recently that a *S. sclerotiorum* adenylate cyclase deletion mutant is incapable of infecting detached host leaves, apparently also as a result of impaired infection-cushion production (Jurick and Rollins 2007). Nonetheless, it is possible that leaf wounding also overcomes impaired lytic enzyme production, another pathogenicity-related trait attributed to necrotrophs (even though preliminary experiments indicate no changes in protease, cellulose, and cutinase secretion in the rgb1 mutants) (A. Erental and O. Yarden, *unpublished*).

This is not the first case of reduced pathogenicity in a *S. sclerotiorum* protein phosphatase mutant. Induction of antisense expression of a type 2B phosphatase (calcineurin) in *S. sclerotiorum* resulted in reduced pathogenesis, apparently as a result of reduced β-1,3-glucan content in the hyphae. Nonetheless, in that case, no inhibition or morphological changes in infection-cushion production were detected (Harel et al. 2006). PP2A recently has been shown to be important in the differentiation process of the protozoan *Giardia lamblia*; because the differentiation of *G. lamblia* in response to physiological stimuli is central to its pathogenesis (Lauwaet et al. 2007), PP2A also may prove to play a role in the pathogenic processes of this organism.

Appressorial melanin limits wall permeability, facilitating osmolyte accumulation and turgor generation within the cell. Without this turgor, the infection peg that protrudes from the adhesive surface of the appressorium cannot mechanically penetrate the underlying host tissue (Henson et al. 1999). Infection cushions are melanin-rich structures and sclerotia are highly melanized; therefore, we postulated that inhibition of PP2A may inhibit the melanin biosynthesis pathway and, consequently, sclerotial maturation and infection-cushion production. The latter clearly is involved in pathogenesis and perhaps a lack of melanin impairs this trait. By screening the *S. sclerotiorum* genome, we identified genes of the DHN melanin biosynthesis pathway which is common to many fungal species (Henson et al. 1999). This pathway initiates with acetate as a precursor. The addition of acetate to the *rgb1* mutant was not sufficient to complement the phenotypic defects in sclerotial maturation or infection-cushion production, suggesting that PP2A also may regulate another downstream enzyme or enzymes in the DHN pathway. It also is possible that PP2A regulates melanin biosynthesis via an additional or alternative route. A likely candidate is the tyrosinase pathway. In fungi, tyrosinases are associated mainly with browning and pigmentation (Halaouli et al. 2006) and tyrosinase activity has been shown to correlate with an increase in the formation of sclerotial initials in *S. sclerotiorum* (Wong and Willetts 1974). Interestingly, a tyrosinase-based melanin biosynthesis pathway in mammalian cells has been suggested to be affected by changes in PP2A and ERK activities (Kim et al. 2005), supporting the possibility that this also may be the case in *S. sclerotiorum*. We have located a putative tyrosinase-encoding gene in *S. sclerotiorum* (SS1G_14461.1), yet its involvement in melanin production in this fungus has not been determined.

Based on our findings, it is tempting to speculate that Smk1 participates in the regulation of PP2A activity in a NOX-dependent manner. Our results indicated that Smk1 positively regulates PP2A activity. However, even though evidence has been provided that PP2A is located upstream of ERK (Fukukawa et al. 2005; Kim et al. 2005; Letourneux et al. 2006), it is still possible that a nonhierarchical cross-talk mechanism regulates part of their activities. The mechanism governing redox effects on phosphatase activity probably is best understood for the protein tyrosine phosphatases (PTPs). All of the PTPs, without exception, contain a highly conserved region of 11 amino-acid residues in their catalytic domain: (Ile/Val)-His-Cys-X-Ala-Gly-X-X-Arg-(Ser/Thr)-Gly. Either oxidation or mutation of the cysteine renders these molecules inactive (Allen and Tresini 2000). However, although PTPs clearly are inhibited via reversible oxidation of cysteine thiols, the function of PP2A as an oxidant-sensitive phosphatase is less clear. Our results, indicating that PP2A activity is positively correlated with NOX activity in *S. sclerotiorum*, imply that the mechanism involved in PP2A regulation may be different from that described for PTPs. Research in mammalian cells has provided evidence that Nox1 expression is activated by the MAPK pathway (Mitsushita et al. 2004); therefore, it is conceivable that, in *S. sclerotiorum*, regulation of NOX by Smk1 occurs at the transcriptional level.

Our current analysis demonstrates the significance of PP2A in the growth, development, and pathogenesis of *S. sclerotiorum*. Expanding the analysis to include additional regulatory subunits along with further elucidation of the roles that MAPK and ROS play in regulating protein phosphatases activity in this pathogen is a rational approach to enhancing our understanding and providing potential targets for antifungal intervention.

MATERIALS AND METHODS

S. sclerotiorum growth conditions.

Wild-type *S. sclerotiorum* isolate 1980 (Godoy et al. 1990) and the *smk1* mutant (Chen et al. 2004) were used in this study. Strains were routinely cultured, unless otherwise stated, on PDA (Difco Laboratories, Detroit). When required, the growth medium was supplemented with hygromycin B (Calbiochem, Riverside, CA, U.S.A.) at 60 μg/ml. When measuring the effect of cantharidin (Sigma-Aldrich, St Louis) on *S. sclerotiorum* development, the fungus was grown on Joham's defined medium (Joham 1943). To test the effect of cantharidin on sclerotial formation, *S. sclerotiorum* was cultured under conditions of nearly synchronous sclerotial formation (Harel et al. 2005) on medium supplemented with different concentrations of cantharidin. Each experiment was performed three times with three replicates for each concentration. To test the effect of cantharidin on sclerotial size, 40 random sclerotia were collected from medium supplemented with 100 μM cantharidin (in three separate experiments) and their width and length determined. When measuring the effect of DPI (A.G. Scientific, San Diego, CA, U.S.A.) on PP2A activity, *S. sclerotiorum* was grown on PDA medium containing 50 μM DPI. To facilitate the harvest of hyphae from cultures grown on solid medium, a

sterile cellophane (Bio-Rad, Hercules, CA, U.S.A.) disk was placed on the medium prior to inoculation. Once the cultures had grown, the disk was removed and the hyphae scraped off for further processing. For antisense induction experiments, cultures were grown in water or on water agar (1.5%) supplemented with either quinic acid (15 mM) or glucose (15 mM).

Nucleic acid isolation and Northern blotting.

Standard recombinant DNA methods were performed according to Sambrook and associates (1989). PCR was performed using the SuperTerm JMR801 Polymerase (Fermentas, Hanover, MD, U.S.A.). RNA extraction was performed by quick-freezing samples in liquid nitrogen followed by grinding the samples with mortar and pestle. Total RNA was extracted with TRI reagent (Sigma-Aldrich). Northern blot analysis was performed according to standard procedures (Sambrook et al. 1989). Total RNA samples (20 μg/lane) were transferred to Magnacharge NT Nylon membranes (MSI, Westborough, MA, U.S.A.). Appropriate RNA probes were generated using the Maxi Script (Ambion, Austin, TX, U.S.A.) kit with an approximately 1-kb fragment of the target gene as template followed by [α-^{32}P] dUTP labeling (Prime-A-Gene; Promega Corp., Madison, WI, U.S.A.). Hybridization was performed at 68°C in the presence of ULTRAhyb solution (Ambion). The most stringent washes were carried out at 68°C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) sodium dodecyl sulfate.

Production of a *pph1* antisense construct.

A fragment of the gene (designated *pph1*) encoding the *S. sclerotiorum* PP2A catalytic subunit (PP2Ac) was cloned by amplifying a 1,085-bp fragment of *pph1* using degenerate primers degF-GARWSNAAYGTNCARCCNGT and degR-RTGYTCRTCDATYTCCATDAT, designed on the basis of the sequence similarity of PP2Ac in *N. crassa, Aspergillus nidulans*, and *M. grisea*. The 1,085-bp fragment of the *pph1* gene (corresponding to nucleotides 228 to 1,313 of the now available annotated gene, designated SS1G_08489.1) was cloned into a pDrive vector (Qiagen, Hilden, Germany). The unique *Sna*bI (pDrive) and *Hin*cII (*pph1*) sites were digested and the excised fragment was ligated into pSO-1 (linearized by *Sma*I) to produce pAE3. pSO-1 was constructed on the basis of a vector described by Fecke and associates (1993) by insertion of the hygromycin phosphotransferase gene (*hph*) as a selectable marker into pWFas51 (S. Oved and O. Yarden, *unpublished data*). The *pph1* antisense construct was used to transform *S. sclerotiorum* according to a standard polyethylene-glycol-mediated transformation protocol (Rollins 2003).

Production of *rgb1* RNAi construct.

A fragment of the gene (*rgb1*) encoding the *S. sclerotiorum* PP2A B regulatory subunit was obtained by amplifying a 1,096-bp fragment, corresponding to nucleotides 566 to 1,662 of *rgb1,* using primers rgbF361-AATTAAGTGGTGCCGAC GAC and rgbR1690-CAACACCTGCAGCTGGACTA, designed on the basis of the sequence of *S. sclerotiorum* that is similar (81% identity at the nucleic-acid level) to the *N. crassa rgb-1* gene. The 1,096-bp *rgb1* fragment was cloned into a pDrive vector (Qiagen) to produce pAE19. The unique *Sna*bI and *Xho*I sites (pDrive) were digested and the excised fragment was ligated into pSilent (Nakayashiki et al. 2005), which was predigested with the same two enzymes to produce pAE20. In the next step, pAE19 was digested with *Kpn*I and *Apa*I and the excised fragment was ligated into pAE20, which was predigested with *Kpn*I and *Apa*I. The resulting construct, pAE21, was used to transform *S. sclerotiorum,* and subsequently isolate pAE21-containing transformants (verified by PCR).

cDNA production and real-time RT-PCR.

RNA was extracted at different stages of sclerotial development (mature sclerotia, hyphae, and white sclerotia) and was purified using the RNeasy Mini Kit (Qiagen). Purified RNA (5 μg) was used for the RT procedures using SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). To analyze the expression levels of *rgb1* transcript, relative quantification of gene expression was performed using SYBR Green Real-Time RT-PCR on an ABI prism 5700 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.). Two specific primers designed for *rgb1* and *tub1*, encoding the PP2A catalytic subunit and β-tubulin control genes, respectively, were used to obtain an amplicon of approximately 100 bp of each target gene. The following primers were designed by utilizing PrimerExpress software (Applied Biosystems): rgbF-CCCGCCTTACTCATCATGACA, rgbR-CATCGCTG TTCACCGAAATG, βtubF-TTGGATTTGCTCCTTTGAC CAG, and βtubR-AGCGGCCATCATGTTCTTAGG. The primers used for β-tubulin amplification were designed on the basis of the *S. sclerotiorum tub1* gene (Harel et al. 2006). RT-PCR mixtures were composed of a 12-pmol concentration of each primer, 12.5 μl of SYBR Green PCR master mix (Applied Biosystems), 5 μl of cDNA (a 1:200 dilution of the 20-μl cDNA product produced as already described), and nuclease-free water to a final volume of 25 μl. Amplification conditions were as follows: 30 min at 48°C, 10 min at 95°C, and then 40 cycles that consisted of 15 s at 95°C and 1 min at 60°C. Total cDNA abundance in the samples was normalized using the *tub1* gene as a control. In all experiments, samples were amplified in triplicate, and the average cycle threshold then was calculated and used to determine the relative expression of each gene. Two independent experiments were carried out in the same manner, and the final average and standard error of the relative expression values were calculated. The experiment results were subjected to *t* test analysis.

PP2A activity assay.

Total protein was extracted from wild-type, PAS1, and *smk1* (PP2Ac and ERK-like MAPK antisense mutants, respectively) strains grown on PDA and transferred, for 24 h, to either an inductive (quinic acid-supplemented) or repressive (glucose-supplemented) water-agar medium prior to extraction. Cell lysate protein concentrations were quantified by the Bradford protein assay (Bradford 1976). PP2A activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate (RII peptide) in the presence or absence of the specific PP2A inhibitor okadaic acid utilizing the cellular assay kit (Biomol, Plymouth Meeting, PA, U.S.A.), with slight modifications. Okadaic acid inhibits both PP2A (0.1 nM < EC_{50} < 1 nM) and PP1 (20 nM < EC_{50} < 100 nM); therefore, we used the concentration of 1 nM, which is known to inhibit mainly PP2A (Fernandez et al. 2002). The amount of liberated PO_4 was determined colorimetrically. Results were normalized on the basis of protein concentration in each sample. The differences in relative (quinic acid/glucose) PP2A activity between the wild-type, PAS1, and *smk1* strains was analyzed by *t* test ($P < 0.05$). Each activity assay was performed using three independent cultures for protein extraction.

Superoxide dismutase and NADPH oxidase activity assays.

For protein extraction, *S. sclerotiorum* cultures subjected to different treatments first were frozen in liquid nitrogen and then ground with mortar and pestle and subsequently homogenized in the presence of extraction buffer consisting of 1 mM DTT, 0.1 mM EGTA, 0.1 mM EDTA, and 50 mM Tris, pH 7.5. The homogenate was centrifuged for 40 min at 12,000 × *g* and the supernatant recovered for SOD and NOX activity assays.

Equal quantities (as determined by Bradford protein assay) of protein extract were utilized for further analysis of SOD and NOX activity. Samples were loaded on 10% native polyacrylamide gels and electrophoresis was performed at 100 to 150 V until the bromphenol blue marker dye had run through most of the gel. Relative in vitro SOD activity was determined on the basis of Beauchamp and Fridovich (1971) by soaking the gels in 100 ml of 0.1 M phosphate buffer, pH 7.8, containing 37 mg of EDTA, 1.25 mg of riboflavin, 20 mg of nitroblue tetrazolium (NBT), and 250 μl of N,N,N′,N′-tetramethylethylenediamine for 30 min, followed by illumination with fluorescent light for 5 to 15 min. During illumination, the gels turned uniformly blue, except where SOD was active. Relative in vitro NOX activity was determined on the basis of a procedure described by Lopez-Huertas and associates (1999). Gels were prepared and run as described above. Following the separation procedure, the gels were incubated in the dark with 2 mM NBT for 20 min and then with 1 mM NADPH until the appearance of blue formazan bands was observed. The reaction was stopped by immersing the gels in distilled water.

Pathogenicity assays.

Seed of *Arabidopsis thaliana* plants, ecotype Columbia-0, were germinated in 0.7% (wt/vol) agar plates containing MS medium (Murashige and Skoog 1962) supplemented with 1.5% (wt/vol) sucrose. Seedlings (3 weeks old) were transferred to Kekkila peat (Tuusula, Finland). Plants were cultivated for an additional 8 weeks and grown under controlled conditions: 22°C, 100 $\mu E/m^2/s$ light intensity for 16 h at 70% relative humidity. Seed of tomato (*Lycopersicon esculentum* Mill. cv. Bonny Best) were planted in Cocos optima (Pelemix, Moshav Katif, Israel) and grown for approximately 6 weeks in the greenhouse at temperatures ranging from 25°C (night) to 30°C (day). Individual, fully expanded leaves of either *A. thaliana* or *L. esculentum* were excised and placed in 9-cm glass dishes lined with water-saturated no. 1 Whatman filter paper. Individual leaves were inoculated with a single 0.3-cm mycelium-colonized agar plug obtained from the expanding margins of PDA-cultured colonies of wild-type or *rgb1* RNAi strains. Inoculated leaves were maintained at 100% relative humidity at 18°C for between 24 and 120 h. When required, leaves were wounded with a sterile botanical needle prior to inoculation. The percentage of infected leaves was determined and differences between treatments analyzed by a χ^2 test. Each experiment was performed with 30 leaves, three times.

Infection-cushion-formation assay.

Mycelium-colonized agar plugs (0.5 cm in diameter) obtained from the expanding margins of PDA-medium-grown colonies supplemented with either wild-type or *rgb1* mutant strains were placed on the surface of empty petri dishes (nine per dish). The petri dishes were maintained at 100% relative humidity and 18°C for 24 h. The formation of infection cushions was monitored by light microscopy using a Zeiss Axioscope microscope.

Oxalic acid quantification.

Cultures of wild-type isolate 1980 or the *rgb1* mutant strain were grown on PDA. When colonies reached the edge of the dish, the fungal mycelium was harvested. Buffered 10 mM EDTA (pH 7.6, 8 ml) was added to the agar medium and the mixture was heated to melt the agar. Samples were cooled to room temperature and 3 ml was mixed with activated charcoal for 5 min. Samples were centrifuged at 1,500 × *g* for 5 min and the supernatant was removed and diluted 10-fold in dilution buffer. The concentration of oxalic acid was determined by the Oxalate Detection Kit (Trinity Biotech, Wicklow, Ireland). Oxalic acid concentration was calculated on the basis of a standard curve (using potassium oxalate) and adjusted for the dilution factors. Each experiment was performed at least twice, using duplicate samples.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Broad Institute *Sclerotinia sclerotiorum* database: www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html