

# Identification and Characterization of *Sclerotinia sclerotiorum* NADPH Oxidases<sup>∇†</sup>

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**Numerous studies have shown both the detrimental and beneficial effects of reactive oxygen species (ROS) in animals, plants, and fungi. These organisms utilize controlled generation of ROS for signaling, pathogenicity, and development. Here, we show that ROS are essential for the pathogenic development of *Sclerotinia sclerotiorum*, an economically important fungal pathogen with a broad host range. Based on the organism's completed genome sequence, we identified two *S. sclerotiorum* NADPH oxidases (SsNox1 and SsNox2), which presumably are involved in ROS generation. RNA interference (RNAi) was used to examine the function of SsNox1 and SsNox2. Silencing of SsNox1 expression indicated a central role for this enzyme in both virulence and pathogenic (sclerotial) development, while inactivation of the SsNox2 gene resulted in limited sclerotial development, but the organism remained fully pathogenic.  $\Delta Ssnox1$  strains had reduced ROS levels, were unable to develop sclerotia, and unexpectedly correlated with significantly reduced oxalate production. These results are in accordance with previous observations indicating that fungal NADPH oxidases are required for pathogenic development and are consistent with the importance of ROS regulation in the successful pathogenesis of *S. sclerotiorum*.**

*Sclerotinia sclerotiorum* is an economically important necrotrophic fungal pathogen with a worldwide distribution of more than 400 dicotyledonous host species (42). Included among these hosts are several agriculturally important plants, including soybean, canola, and sunflower (15). *S. sclerotiorum* is a particularly devastating pathogen, in part due to the ineffectiveness of traditional control measures. Spray regimes have been largely unsuccessful, and breeding programs have had limited success, presumably since resistance is genetically complex. Crop rotation strategies are hampered by the long-term viability (years) of sclerotia and the inherent broad host range of the fungus. Thus, while considerable effort has been made to prevent *Sclerotinia* diseases, there are currently no effective control regimes. As the development and pathogenesis of *S. sclerotiorum* are complex and not well understood, intervention with the disease cycle of this pathogen has proven difficult.

We are interested in identifying the factors that are responsible for or contribute to the pathogenic success of this fungus. Work from our lab as well as those of others has established that a primary determinant contributing to the pathogenic success of this fungus is its ability to synthesize and secrete high concentrations (~10 mM) of oxalic acid (OA). Mutants of this fungus that are defective in OA production are nonpathogenic (13). We have extensively investigated the function of OA in the pathogenicity of this fungus (4, 5, 6, 17, 29, 30, 41).

Oxalate was proposed to enhance *S. sclerotiorum* virulence

in at least three ways (7). First, oxalate reduces pH. Several cell wall-degrading enzymes secreted during invasion of plant tissues (e.g., polygalacturonase) have maximal activities at low pHs (2). Second, oxalate may be directly toxic to host plants (27). Finally, chelation of cell wall Ca<sup>2+</sup> by the oxalate anion has been proposed both to compromise the function of Ca<sup>2+</sup>-dependent defense responses and to weaken the plant cell wall (2).

In recent years, we have shown that OA has several unique functions that contribute to the success of this phytopathogen. For example, OA is an elicitor of reactive oxygen species (ROS)-induced plant programmed cell death (17) but, paradoxically, can also dampen the host oxidative burst (4). We have recently shown that during the initial stages of colonization, *Sclerotinia* (via OA) generates reducing conditions that suppress host defense responses, including the oxidative burst and callose deposition, akin to the actions of hemibiotrophic pathogens (41). Once infection is established, however, *Sclerotinia* induces the generation of plant reactive oxygen (ROS) and programmed cell death, which is of direct benefit to this necrotrophic pathogen. In contrast, a nonpathogenic OA-deficient mutant failed to alter host redox status or these defense responses. We have concluded that ROS regulation and signaling play a key role in the pathogenicity of *S. sclerotiorum* and, thus, that interference with this process is a means to impair pathogenicity. ROS regulation is also known to play an important role in sclerotial differentiation (11), a key function in the development and pathogenicity of this fungus. We therefore functionally investigated the *S. sclerotiorum* NADPH oxidase (Nox) family, whose members are established generators of ROS.

NADPH oxidases are a primary source for generating superoxide, an important precursor of several ROS, including

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hydrogen peroxide. For the activation of Nox enzymes, cytosolic regulatory components (Rac, p67<sup>phox</sup>, p47<sup>phox</sup>, and p40<sup>phox</sup>) are recruited into the integral membrane protein flavocytochrome b558, consisting of the catalytic subunits gp91<sup>phox</sup> and p22<sup>phox</sup> (26, 32). The activated Nox enzyme complex generates superoxide from oxygen by utilizing NADPH as an electron donor, thereby leading to the formation of other species of reactive oxygen. Among NADPH oxidases in animals, the most well known is Nox2 (gp91<sup>phox</sup>), which is involved in several diseases (33, 32). As in animals, the plant NADPH oxidase Rboh (respiratory burst oxidase homolog), the gp91<sup>phox</sup> homolog, is considered to be a primary, but not the only, source of ROS (39). ROS from the *Arabidopsis* NADPH oxidases (AtrbohD and AtrbohF) are associated with host defense via the hypersensitive response (39), possibly by antagonizing salicylic acid-dependent prodeath signals (40). ROS-dependent abscisic acid (ABA) signaling by these enzymes also regulates cytosolic Ca<sup>2+</sup> levels, thereby controlling stomatal closure, seed germination, and root elongation (19). In addition, an *Arabidopsis* NADPH oxidase (AtrbohC) regulates plant cell expansion through the activation of Ca<sup>2+</sup> channels during root hair development (10).

NADPH oxidases have been found in most fungal species, excluding the hemiascomycete yeasts and other unicellular fungi (36). Interest in fungal NADPH oxidases has recently increased. Several studies have shown the importance of fungal NADPH oxidases in sexual and asexual development (3, 22, 23). In several plant-pathogenic fungi, deletion of *nox* genes resulted in developmental defects in structures that are key for infection, such as appressoria and sclerotia (8, 34). It has also been shown in the symbiotic interaction between the endophytic fungus *Epichloë festucae* and perennial ryegrass that ROS generated from fungal NADPH oxidases function to modulate plant-fungus symbiosis (37).

This study examined the contribution of *S. sclerotiorum* NADPH oxidases to pathogenesis, growth, and development. We identified two predicted NADPH oxidase genes in the *S. sclerotiorum* genome sequence (*Ssnox1* and *Ssnox2*) (<http://www.broadinstitute.org>). Following the cloning and characterization of *Ssnox1* and *Ssnox2*, these genes were functionally evaluated by RNA interference (RNAi)-mediated gene silencing. Both of these genes were found to be required for sclerotial development. In addition, *Ssnox1* silenced mutants were severely compromised in their ability to cause disease. We also noticed a significant reduction in the production of oxalate by *Ssnox1* mutants. Tomato plants challenged with the *Ssnox1* strain showed an increased plant oxidative burst, suggesting that the decreased virulence of the *Ssnox1* strain may result in an increased or active defense response in host plants that does not occur in wild-type strains. *Ssnox2* mutant-infected plants showed negligible decreases in the generation of superoxide and were still pathogenic. Hence, *Ssnox1* of *S. sclerotiorum* is important for both virulence and fungal development and is correlated with oxalate production.

#### MATERIALS AND METHODS

**Fungal strains, growth conditions, and media.** The *S. sclerotiorum* wild type (strain 1980) and Nox silenced mutants were incubated on potato dextrose agar (PDA) or broth (PDB) at 25°C. Infections were conducted on detached tomato (Rutger) leaves. For pharmacological studies, wild-type *S. sclerotiorum* was in-

cubated on PDA for 2 to 3 days. Agar plugs were then transferred to PDA containing 40 mM *N*-acetyl-cysteine (NAC) or 1 mM diphenyleiiodonium (DPI), and growth and sclerotial development were monitored over time. Oxalic acid levels were determined after growth of the designated *S. sclerotiorum* strains on PDA or PDB. After incubation, 10 mM EDTA (pH 7.6) was added to the agar plug, and the mixture was melted at 95°C. Samples in the PDB were directly analyzed. Oxalic acid was quantified using an oxalate detection kit according to the manufacturer's recommendations (Trinity Biotech, Wicklow, Ireland).

**RNAi constructs and fungal transformation.** The fungal RNAi vector pSilent-1 was obtained from Oded Yarden. Sequences of *S. sclerotiorum* Nox1 and Nox2 were obtained by aligning the human gp91<sup>phox</sup> amino acid sequence with the sequence of the *S. sclerotiorum* genome ([http://www.broadinstitute.org/annotation/genome/sclerotinia\\_sclerotiorum/MultiHome.html](http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html)). *nox1* and *nox2* were amplified from *S. sclerotiorum* genomic DNA and cloned into pGEM-T. To silence *nox1*, sense and antisense fragments were amplified using two sets of primers (XhoINOX1F [AAC TCGAGCGAAAGCCATCGATGAAG] and HindIIIINOX1R [AAAAGCTTCG ACATCGGCTCTACAC] and KpnINOX1F [AAGGTACCCGAAAGCCATCG ATGAG] and BglIIINOX1R [AAAGATCTCGACATCGGCTCTACAC]) and introduced into pSilent-1 with a spacer fragment to make a hairpin RNA structure. To silence *nox2*, sense and antisense fragments were amplified using two sets of primers (XhoINOX2F [AACTCGAGTGCTAGATCAGCT GCCTTGA] and HinIIIINOX2R [AAAAGCTTCCTCGGACAACAAAAG AAA] and KpnINOX2F [AAGGTACCTGTAGATCAGCAGCCTTGA] and BglIIINOX2R [AAAGATCTCTCGGACAACAAAAGAAA]), and PCR sets were used to construct the silencing vector. These silencing vectors for *nox1* and *nox2* and the pSilent-1 vector control were used to transform *S. sclerotiorum*. Generation of protoplast and fungal transformation was performed as described by Rollins (31). Total RNA was extracted with TRIzol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

**Semiquantitative RT-PCR analyses.** For semiquantitative reverse transcription-PCR (RT-PCR), total RNA was DNase treated and subjected to first-strand DNA synthesis using Moloney murine leukemia virus (MMLV) reverse transcriptase. Nox1 and Nox2 were amplified using SsNOX1F (CGAAAGCCATCG ATGAAG) and SsNOX1R (CGACATCGGCTCTACAC) and SsNOX2F (T GCTAGATCAGCTGCCTTGA) and SsNOX2R (CCTCGGACAACAAA GAAA), respectively. As an internal control, SsEF-1F (TCCTATCTCCGGTT TCAACG) and SsEF-1R (GCAAGCAATGTGAGCAGTGT) were used.

**ROS detection assay.** In order to detect superoxide in tomato leaves (1 to 2 days after fungal inoculation), leaves were placed in 0.5 mg/ml nitroblue tetrazolium (NBT) (10 mM potassium phosphate buffer, pH 7.5) aqueous solution for 2 h. After incubation, leaves were rinsed in 70% ethanol and mounted in 50% glycerol.

For detection of superoxide in fungal structures, tissues were incubated in 0.5 mg/ml NBT (10 mM potassium phosphate buffer, pH 7.5) aqueous solution for 2 h. After incubation, fungal tissues were observed with light microscopy. A colorimetric NBT assay was also performed. After incubation with NBT, fungi were ground in liquid nitrogen. The reduced NBT was solubilized with the same volume of 2 M KOH and a 1.3× volume of dimethyl sulfoxide (DMSO) for 30 min.

ROS staining was conducted on agar plugs of the wild-type isolate 1980 shortly after sclerotial initials were observed. Aliquots of protoplasts generated from sclerotial initials were then stained with 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) to visualize H<sub>2</sub>O<sub>2</sub>.

**Microscopy.** Microscopic observations were made using an Olympus IX-81 microscope with differential interference contrast optics (10× UPlanFL N objective, 0.30 numerical aperture [NA]). Digital images were captured with an Olympus DP70 camera. All images were collected using Olympus DP controller and manager software under the same conditions (image size, 1,360 by 1,024; exposure time, 60.0 s; ISO sensitivity, ISO200; exposure mode, auto) and were saved as TIF files.

## RESULTS

**Generation of ROS by *S. sclerotiorum* is necessary for sclerotial development.** The sclerotium is a multicellular, highly melanized structure for overwintering and long-term survival and plays an important role in disease propagation. Since it has been postulated and later shown that ROS levels impact fungal development (11), we monitored intracellular ROS production from sclerotial initials using the oxidant-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCHF-

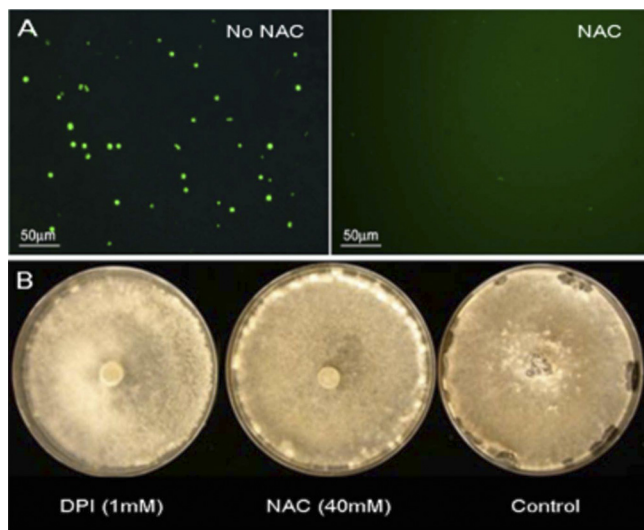


FIG. 1. Antioxidants impair sclerotial development in *S. sclerotiorum*. (A) Sclerotial initials generate large amounts of ROS. An agar-mycelium plug of the wild-type isolate 1980 was inoculated onto potato dextrose agar (PDA) and grown until the appearance of sclerotial initials. Aliquots of protoplasts generated from sclerotial initials were then stained with 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) to visualize ROS within the cells in response to *N*-acetyl-cysteine (NAC), as described in Materials and Methods. Images shown are representative of three independent experiments. (B) An agar-mycelium plug of the wild-type isolate 1980 was inoculated on PDA with or without 40 mM NAC or 1 mM diphenyleneiodonium (DPI). Sclerotial development was monitored and photographed.

DA). Fluorescence emission was higher in sclerotial initials, suggesting that sclerotial initiation is associated with generation of intracellular ROS (Fig. 1A). Inhibition of ROS is thus predicted to negatively impact sclerotial development. Indeed, when wild-type *S. sclerotiorum* was treated with 40 mM *N*-acetyl-cysteine (NAC), an effective antioxidant and ROS scavenger, or 1 mM diphenyleneiodonium (DPI), an inhibitor of flavoenzymes such as NADPH oxidase, sclerotial initial formation was considerably reduced and sclerotial maturation was prevented (Fig. 1B). Similar results were also reported with *Botrytis cinerea* grown on DPI (34). There were no significant differences in the extents of the radial growth of *S. sclerotiorum* under these conditions (data not shown). Together, these experiments suggest that ROS generation and signaling is required for proper development, including sclerotial formation, and that an NADPH oxidase may play an important role in this signaling pathway.

**Transcript levels of *S. sclerotiorum* Nox1 increase in planta and during sclerotial development.** From the available, completed genomic sequence of *S. sclerotiorum*, we identified two predicted *nox* genes that harbored significant homology to human gp91<sup>phox</sup> and Nox homologs in several filamentous fungi. These two *nox* genes were amplified from genomic DNA of *S. sclerotiorum*. The predicted amino acid (aa) sequences of *S. sclerotiorum* Nox1 (541 aa) and Nox2 (581 aa) were aligned with *Epichloë festucae* NoxA (GenBank accession no. AB236860.1), *E. festucae* NoxB (AB236861.1), *Aspergillus nidulans* NoxA (AY174088.1), *Podospora anserina* Nox1 (AF364817.1), *P. anserina* Nox2 (AY372210.1), *Neurospora*

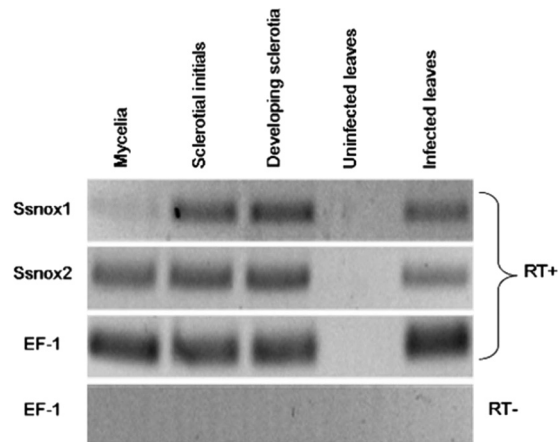


FIG. 2. Expression analysis of Nox genes of *S. sclerotiorum* during development and plant infection. RNA was extracted from fungal and leaf tissues in the indicated stages, 4 days after inoculation with an agar plug (5 mm in diameter) colonized with *S. sclerotiorum* or uncolonized PDA. RT-PCR was performed with gene-specific primers for SsNox1, SsNox2, or elongation factor 1 $\alpha$  (EF-1) as described in Materials and Methods. RT<sup>-</sup>, no-template control.

*crassa* NoxA (AABX02000003.1), and *N. crassa* NoxB (AABX02000010.1). The *Ssnox1* sequence is 74%, 69%, 73%, and 72% homologous to *E. festucae* NoxA, *A. nidulans* NoxA, *N. crassa* NoxA, and *P. anserina* Nox1, respectively. SsNox2 is 77%, 78%, and 79% homologous to *E. festucae* Nox2, *N. crassa* NoxB, and *P. anserina* Nox2, respectively. A 36% similarity level was noted between *Ssnox1* and *Ssnox2*. Both *S. sclerotiorum* Nox coding regions contain hallmark features of NADPH oxidases, including a six-transmembrane (TM) region, a flavin adenine dinucleotide (FAD) binding motif, and four NADPH binding motifs (see Fig. S1 in the supplemental material). Since these are highly conserved regions among multicellular organisms, it is expected that the two NADPH oxidases of *S. sclerotiorum* function similarly to other fungal homologs.

Semiquantitative RT-PCR was used to examine the expression levels of *S. sclerotiorum nox* genes during sclerotial development *in vitro* and in disease development in tomato leaves. *Ssnox1* and *Ssnox2* showed distinct patterns of expression during both processes. *Ssnox2* was constitutively expressed throughout sclerotium formation, as well as during interaction with the plant, showing no detectable expression changes (Fig. 2). However, transcript levels of *Ssnox1* were low during hyphal growth and dramatically increased during the induction of sclerotial initials and developing sclerotia. Furthermore, transcript levels of *Ssnox1* also increased during fungal interaction with plant tissue (Fig. 2). These data show that *Ssnox1* and *Ssnox2* expression patterns are not redundant and that their functions are likely distinct but partially overlap. Importantly, *Ssnox1* appears to play a more important role than *Ssnox2* in disease development and progression.

***Sclerotinia nox* RNAi mutants.** RNA interference (RNAi)-mediated gene silencing has proven to be an efficient method to inhibit the expression of genes in many eukaryotes, including fungi (25). To genetically investigate the role of *Ssnox1* and *Ssnox2* in development and pathogenesis, the RNAi silencing vector pSilent-1 (25) was used to express hairpin structures of



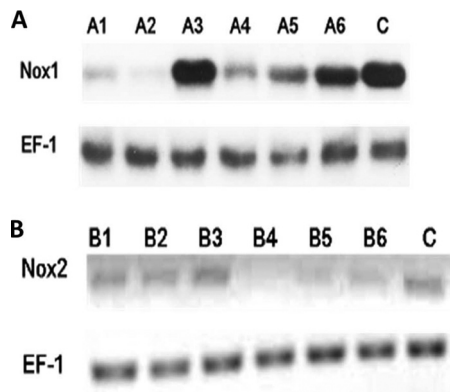


FIG. 3. Silencing of *S. sclerotiorum nox1* and *nox2*. (A) A Northern blot to determine Nox1 mRNA expression was analyzed with the Nox1 silenced transformants. Elongation factor-1 (EF-1) served as an internal control. A1, A2, A4, and A5 (silenced transformants) show reduced RNA expression compared to the wild-type control and nonsilenced transformants (A3 and A6). (B) RT-PCR analysis of Nox2 mRNA expression in the Nox2 silenced transformants. EF-1 served as an internal control. B1 to B3 are nonsilenced transformants, and B4 to B6 are silenced transformants. C, wild-type control.

*Ssnox1* and *Ssnox2*. We constructed two silencing vectors (pSNOX1 and pSNOX2) carrying the hygromycin B resistance gene as the selectable marker. As a control, an empty vector (pSilent-1) was used. These constructs were introduced into wild-type *S. sclerotiorum* via protoplast transformation (31). RNA blots and semiquantitative RT-PCR analyses (Fig. 3) were used to confirm silencing in the transformants. *Ssnox1* (A2) and *Ssnox2* (B4) silenced mutants were selected for further study (Fig. 3).

To examine the role of *nox* genes during hyphal growth, the *nox* silenced mutants, the empty vector control, and the wild-type isolates were grown on potato dextrose agar (PDA). Extents of hyphal growth were not significantly different in all strains (Fig. 4). However, closer examination revealed that hyphae of the *Ssnox2* mutant were highly branched compared to those of the wild-type, vector control, and *Ssnox1* strains (Fig. 5). The *Ssnox2* branching is reminiscent of the previously reported *E. festucae noxA* deletion mutant phenotype (37). Taken together, this suggests that NADPH oxidases may be involved in the regulation of hyphal branching.

To determine whether sclerotial development was affected in these mutant strains, we monitored sclerotium formation on PDA. Consistent with the pharmacological study,  $\Delta Ssnox1$  mutants were unable to produce sclerotia, and only occasionally were *Ssnox2* mutants able to produce sclerotia (Fig. 4). In contrast, wild-type *S. sclerotiorum* and strains carrying the empty vector showed normal sclerotial development. The defect in sclerotial development of Nox silenced strains was not due to aberrant physiological growth defects since the extents of radial growth of all strains were nearly identical (Fig. 5). Together with the results of DPI/NAC treatments, these data support the requirement of NADPH oxidases for proper sclerotial development.

**Production of superoxide in the  $\Delta Ssnox1$  mutant is impaired.** NADPH oxidases generate superoxide. Therefore, we reasoned that in a *nox* mutant background, the accumulation of superoxide is likely to be reduced. To determine whether

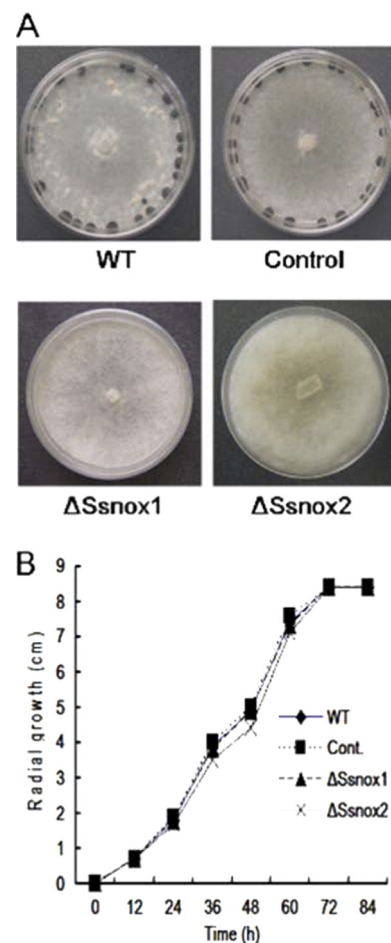


FIG. 4. Phenotypes of *nox* knockdown mutants of *S. sclerotiorum*. (A) Sclerotial development of Nox silenced mutants ( $\Delta Ssnox1$  and  $\Delta Ssnox2$ ) on PDA. Defects in sclerotial development were observed in the  $\Delta Ssnox1$  and  $\Delta Ssnox2$  mutants, while the wild type (WT) and a transformant (control) containing an empty vector developed only sclerotia. Photographs were taken 3 weeks postinoculation. (B) Comparison of hyphal growth rates on PDA. Agar plugs (5 mm in diameter) obtained from growing hyphal tips of each tested strain were inoculated at the center of the petri dish. Colony diameters were measured at 12-h intervals. Cont., control. Data were obtained from three independent assays; error bars are omitted for clarity.

inactivation of SsNox1 and SsNox2 leads to altered ROS generation, we examined ROS production during hyphal growth and sclerotium formation in both mutants. Nitroblue tetrazolium (NBT) staining was used to detect superoxide (4). The wild-type and Nox silenced mutant strains were cultured on PDA and stained with NBT 4 days later, when sclerotium formation initiates. Dark-blue formazan precipitates were observed in the wild-type fungus during hyphal growth/sclerotial initiation (Fig. 6). During hyphal growth, no significant differences in superoxide production were observed between *Ssnox2* silenced mutants and the wild type, whereas the *Ssnox1* silenced mutant had a significant reduction in superoxide accumulation (Fig. 6). This significant reduction in staining indicates that superoxide production was impaired in this mutant. Generation of superoxide in *Ssnox2* mutants was also reduced, but not to the extent observed in *Ssnox1* mutants (Fig. 6).

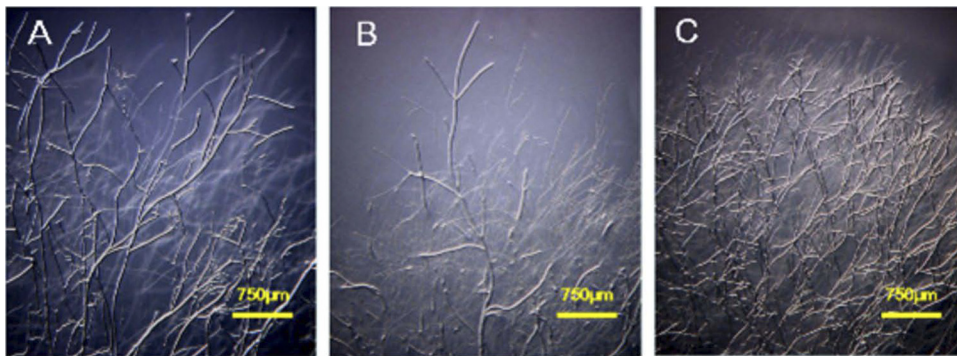


FIG. 5. *In vitro* hyphal development of  $\Delta Ssnx1$  and  $\Delta Ssnx2$  mutants. The two Nox silenced mutants and wild-type strains were cultured on PDA for 2 days. Vegetative hyphae were observed under a dissecting microscope. (A) Wild type; (B)  $\Delta Ssnx1$  mutant; (C)  $\Delta Ssnx2$  mutant. Bars = 750  $\mu\text{m}$ .

**The SsNox1 mutant is compromised in virulence, and oxalate production is significantly reduced.** We were interested in determining the phenotypes of our mutant strains with respect to pathogenicity. NADPH oxidase mutants of *Magnaporthe oryzae* and *Botrytis cinerea* have been shown to be less pathogenic than the corresponding wild-type fungal strains (8, 34). In accordance, the virulence of the *Ssnox1* mutant in tomato plants was also found to be significantly reduced. However, the *Ssnox2* mutant was as virulent as the wild type (Fig. 7). This correlated with the results from the RT-PCR analysis of SsNox genes showing that SsNox1 expression was induced during infection and that SsNox2 levels were not altered and were constitutively expressed (Fig. 2). Therefore, SsNox1 appears to play a more prominent role during infection and pathogenic development and, as will be discussed below, is also linked to oxalate production.

Recent studies in *Aspergillus* species showed that the accumulation of ROS was linked to the production of the secondary metabolite aflatoxin (16, 28). Based on this result and the well-established importance of disease development with the

multifunctional secondary metabolite oxalic acid, we asked whether reduced levels of ROS in the *Ssnox1* mutant affects oxalate production. Oxalate levels were measured in both the mutant and wild-type strains at 3 days postculturing in potato dextrose broth (PDB). The  $\Delta Ssnox1$  mutant produced about half as much oxalate as wild-type *S. sclerotiorum* (Fig. 8), although not to the extent of the  $\text{OA}^- \text{A2}$  mutant (4). However, the *Ssnox2* mutant's OA levels were approximately the same as those of the wild type (Fig. 8). Oxalate production was then determined during fungal interaction with the plant. At 2 days postinfection, plugs were removed from the leaf tissue and oxalate levels determined. Consistent with *in vitro* data, the production of oxalate was reduced in the *Ssnox1* (nonpathogenic) mutant similarly to in the wild type during host challenge. The *Ssnox2* mutant showed no difference in its production of oxalate from that of the wild type and was equally pathogenic (Fig. 7 and 8). Importantly, when the *Ssnox1* mutant was supplemented with potassium oxalate (KOX; pH 7.0), though not complete, an increase in virulence was observed. This treatment had no effect on wild-type virulence (Fig. 9).

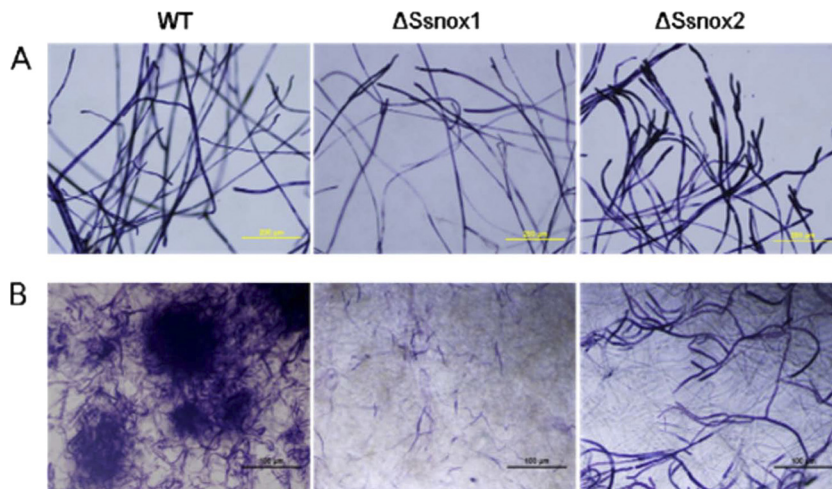


FIG. 6. Superoxide accumulation in Nox silenced mutants. Strains were inoculated on PDA. Superoxide accumulation was observed at 4 days postinoculation (sclerotial initial). For detection, NBT staining was used and the intensity was monitored under a light microscope. Blue staining represents the accumulation of superoxide. The wild type shows extensive precipitation of blue formazan, and the  $\Delta Ssnox1$  and  $\Delta Ssnox2$  mutants show lesser accumulations of blue formazan. Bars = 100  $\mu\text{m}$ .

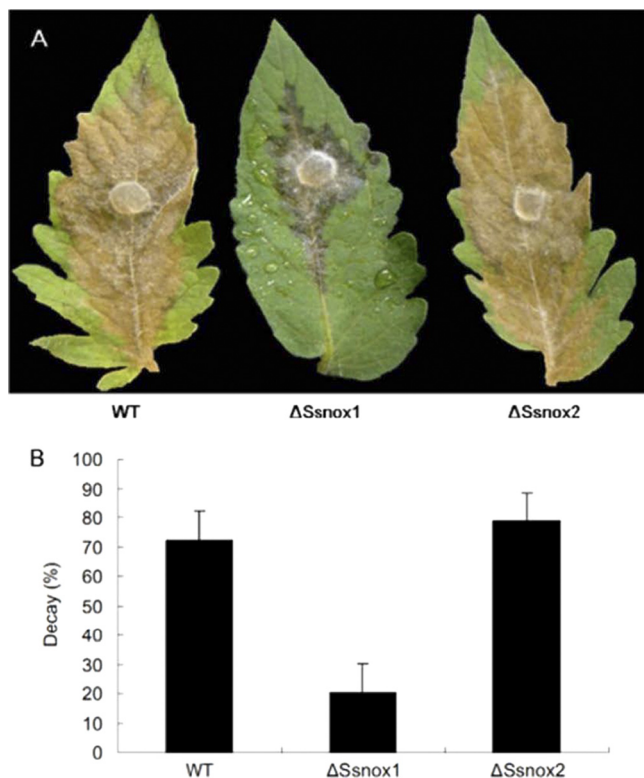


FIG. 7. Pathogenicity of the  $\Delta Ssnx1$  and  $\Delta Ssnx2$  RNAi mutants. (A) Lesion development on tomato leaves. Leaves were inoculated with an agar plug (5 mm in diameter) of each strain. Inoculation with wild-type fungus served as the control. Photographs were taken at 3 days after inoculation. (B) Relative lesion areas on tomato leaves. Lesion areas in panel A were quantitatively analyzed with ImageJ 1.38x software.

KOH alone had no detectable effects on the leaf for the duration of this experiment (data not shown). These observations suggest a direct correlation between ROS generation, oxalate, and disease development. Since DPI blocks sclerotium formation (Fig. 1B), we examined the production of oxalate by growing fungi in PDB treated with DPI (0 to 1 mM) (Fig. 10). Results showed that DPI treatment inversely correlated with the production of oxalate in a dose-dependent manner. When ROS decreased, levels of oxalate also decreased. This further suggests that NADPH oxidases are linked to oxalate production, consistent with the limited ability of the fungus to cause disease.

## DISCUSSION

A growing body of evidence has established that reactive oxygen species flux and redox regulation have key regulatory functions with respect to fungal disease development and host plant-resistant responses (3, 8, 12, 22, 23, 34, 36, 37). A principal means by which ROS are generated is via NADPH oxidases. From the available genome sequence of *S. sclerotiorum*, we identified two homologs of human gp91<sup>phox</sup> in *S. sclerotiorum*. Based on data reported thus far, filamentous fungi generally have two Nox subfamilies (NoxA/Nox1, NoxB/Nox2), while others, including *Fusarium* species, *M. oryzae*, *P. an-*

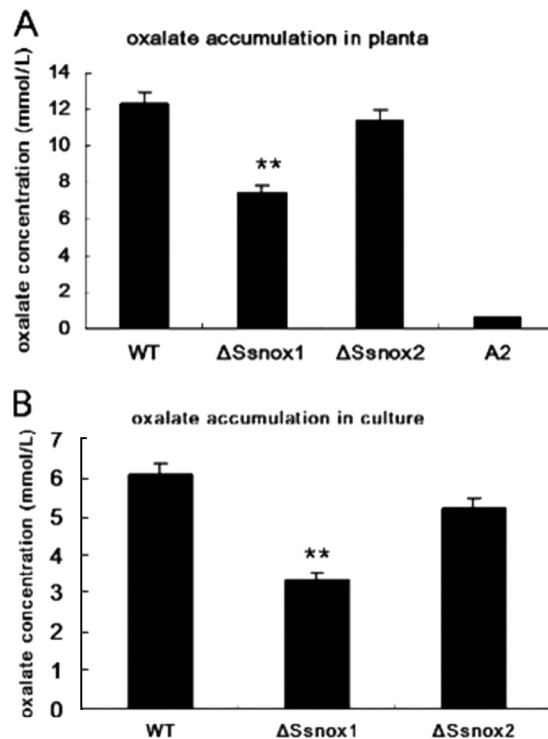


FIG. 8. Oxalate accumulation in the  $\Delta Ssnx1$  and  $\Delta Ssnx2$  mutants. (A) Oxalic acid accumulation *in planta*. Leaves were inoculated with a PDA plug (5 mm in diameter) colonized with the indicated strains. Wild-type fungus served as a positive control, and an oxalate-deficient mutant (A2) was used as the negative control. (B) Oxalic acid production in potato dextrose broth (PDB). Each strain was cultured for 3 days, and the resulting liquid culture was analyzed for oxalate accumulation.

*serina*, *Aspergillus terreus*, and *Phaeosphaeria nodorum*, have a third Nox subfamily member (NoxC/Nox3). NoxA/Nox1 and NoxB/Nox2 are homologous to human gp91<sup>phox</sup>, whereas NoxC/Nox3 has a predicted calcium binding elongation factor



FIG. 9. Oxalate partially restores the virulence of the Nox1 silenced mutant. *Nicotiana benthamiana* leaves were infiltrated with either water or potassium oxalate (KOX) and then immediately inoculated with agar plugs colonized with wild-type *S. sclerotiorum* or the  $\Delta Ssnx1$  mutant. 1, wild type plus water; 2, wild type plus KOX (pH 7.0); 3,  $\Delta Ssnx1$  mutant plus water; and 4,  $\Delta Ssnx1$  mutant plus KOX (pH 7.0) at room temperature. Lesion development was monitored over 2 days. Photographs were taken 1.5 days postinoculation.



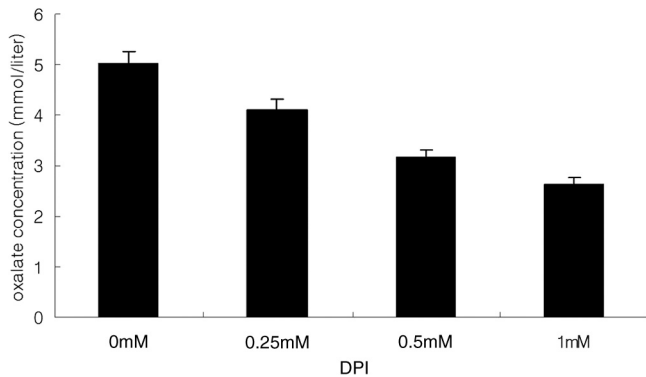


FIG. 10. Oxalate accumulation and ROS inhibition following DPI treatment. *S. sclerotiorum* wild-type isolate 1980 was cultured in PDB treated with 0 mM to 1 mM DPI and analyzed for oxalate accumulation.

(EF)-hand motif in the NH<sub>2</sub> termini also found in mammalian Nox5 and plant Rboh proteins (32).

In this study, we identified and characterized the two predicted *nox* genes (*Ssnox1* and *Ssnox2*) in *S. sclerotiorum*. Both *S. sclerotiorum* Nox coding regions contain a six-transmembrane (TM) region, a putative FAD binding motif, and four NADPH binding motifs similar to those of plant and animal NADPH oxidases. However, neither SsNox protein has a putative calcium binding EF-hand motif at its NH<sub>2</sub>-terminal end, consistent with the membership of its gene in the two-member subgroup of fungal Nox genes. Increased ROS has been observed in several fungal developmental structures, including cleistothecia, appressoria, and hyphal tips (8, 22, 23, 36). Similarly, generation of ROS was observed in sclerotial initials and infection cushions in *S. sclerotiorum*. To more directly assess the formation of reactive oxygen metabolites, we determined intracellular peroxide levels with DCHF-DA, which fluoresces when oxidized. Protoplasts generated from sclerotial initials treated with DCHF-DA were specifically fluorescent (vegetative hyphae were not), correlating ROS production with development. Consistent with this observation, the ROS scavenger NAC or the NADPH oxidase inhibitor DPI prevented DCF fluorescence and blocked sclerotial formation. Georgiou and colleagues (11) have also shown that hydroxyl radical scavengers and antioxidants inhibit the sclerotial development of several fungi, including *S. sclerotiorum*. Antioxidants, including *N,N'*-diphenyl-1,4-phenylene diamide, 1,3-dimethyl-2-thiourea, ammonium pyrrolidine dimethyl-dithiocarbamate, and NAC, blocked chemiluminescence enhanced by lucigenin (indicating the formation of ROS) during the differentiation process and conidiation of *Neurospora crassa* (14), similar to our observations. Exposure to the antioxidant ascorbic acid and the catalase mimic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin also inhibited germination and appressorium formation of *M. oryzae* (8). Taken together, our data coupled with previous reports indicate the importance of ROS signaling for various fungal developmental structures.

To genetically examine the two *S. sclerotiorum nox* genes, we utilized an RNAi approach. We produced several knockdown mutations in both genes. Silencing *Ssnox1* resulted in strains unable to form sclerotia. In other fungi, NoxA/Nox1 was shown

to be involved in the development of sexual structures. For example, in *A. nidulans*, NoxA knockout mutants are defective in the differentiation of cleistothecia, although hyphal growth and asexual development were normal (22). Studies with *N. crassa* and *Podospora anserina* also showed a block in sexual development when Nox1 homologs were deleted. In contrast to NoxA of *A. nidulans*, Nox1 of *N. crassa* is also involved in asexual development and hyphal growth (3, 23). In *S. sclerotiorum*, asexual sclerotium formation is required for generation of the apothecium, a fungal sexual fruiting body. Thus, *Ssnox1* RNAi mutants are unable to develop apothecia via their inability to form the necessary sclerotial precursor. In contrast to NoxA/Nox1, NoxB/Nox2 is directly required for ascospore germination in *N. crassa* and *P. anserina* (3, 23). Importantly, this result is analogous to what was reported for the closely related necrotroph *Botrytis cinerea*, where both *nox* genes were involved in sclerotial formation. *B. cinerea noxB* mutants produced few, small, and abnormal sclerotia and failed to develop apothecia (34). Transcript levels of *Sclerotinia* NADPH oxidases also correlated with ROS generation in the *nox* silenced mutants. Generation of ROS in the *Ssnox1* was significantly reduced during sclerotial development but not in the *Ssnox2* mutants. These results suggest that *Ssnox1* contributes to the induction of ROS in sclerotial initials and that *Ssnox2* plays a minimal role in the accumulation of ROS during sclerotial development. The data presented show that *Ssnox* genes and ROS signaling are key determinants in *S. sclerotiorum* development and pathogenicity.

**NADPH oxidase activity is associated with virulence.** Egan and colleagues demonstrated that the *Magnaporthe oryzae nox1* and *nox2* genes play a pivotal role in appressorium differentiation, a specialized infection structure required for fungal penetration into host tissue (8). Nox-generated ROS within the *M. oryzae* appressorium facilitates oxidative cross-linking of proteins, thereby strengthening the appressorium cell wall to withstand the enormous turgor pressure generated during breaching of the host cuticular barrier (38). Deletion of *nox* genes resulted in weakened appressorial cell walls and the inability to penetrate and cause infection (8). Consistent with these findings, BcNox2 is similarly required for appressorium penetration of the plant cuticle in *B. cinerea* (34). In our study, silencing of *nox1* significantly reduced virulence in *S. sclerotiorum*, while *Ssnox2* silenced mutants were similar to the wild type with respect to virulence. Recent studies with *Epichloë festucae*, a fungal mutualist of ryegrass, provided interesting and, at first glance, perhaps unexpected evidence for involvement of ROS in a plant-fungal mutualistic relationship (36, 37). A restriction enzyme-mediated integration (REMI/knockout) screen revealed that one of the two *E. festucae nox* genes (NoxA), when interrupted, resulted in the conversion from a mutualistic to a pathogenic lifestyle (37). Thus, while wild-type *E. festucae* exhibits a symbiotic association with the perennial ryegrass, inactivation of *E. festucae* NoxA resulted in pathogenicity, increased fungal biomass, and disease (37). Importantly, ROS accumulation was detected at the interface between the extracellular matrix and host cell walls of meristematic tissue in the interaction of ryegrass and *E. festucae* (wild type) (37). Further, this group showed a decrease in ROS accumulation at the interface between ryegrass and the *noxA* mutant (37). These observations suggest that wild-type *E. festucae* may re-

quire ROS (generated from NADPH oxidase) for signaling in the host plant (37). This ROS signal from wild-type *E. festucae* may moderate the plant defense response to maintain mutualism. It will be of particular interest to unravel the plant components that are modulated by fungal ROS to promote this phenotype. Though the interactions between *S. sclerotiorum* and *E. festucae* with their respective hosts differ in outcome, both organisms utilize similar strategies involving *nox* regulation to subdue defenses and gain unimpeded entry into the host plant.

**NADPH oxidases and oxalate production.** During the course of these studies, we noticed that *Ssnx1* expression is linked to oxalate production. As discussed, OA is an established pathogenicity factor in *S. sclerotiorum* (4, 6, 13). Oxalate levels in *Ssnx1* mutants were significantly lower than in wild-type strains and *Ssnx2* mutants during *in vitro* culture and, importantly, during interaction with plant tissue. Further, DPI, an inhibitor of NADPH oxidase, directly reduced the production of oxalate. Thus, genetically and biochemically, ROS are linked to oxalate and both are linked to virulence. In *Aspergillus*, the generation of ROS correlates with the production of the secondary metabolite aflatoxin (28, 32). Moreover, exogenous application of the antioxidant caffeic acid significantly reduced the production of aflatoxin (16). In studies from our lab, a superoxide dismutase (SOD) mutant of *S. sclerotiorum* also exhibited reduced levels of oxalate production and reduced virulence (S. Veluchamy, K. S. Kim, B. Williams, and M. B. Dickman, unpublished data). These observations are consistent with the established link between NADPH oxidase, superoxide generation, and activation of SOD (32). Superoxide generated from NADPH oxidases is converted to hydrogen peroxide via SOD. Therefore, the similar decreases in net oxalate production when NADPH oxidase and SOD are impaired suggest that these two enzymes function in the same pathway. Importantly, addition of OA to *Ssnx1* mutants increased virulence (Fig. 9).

An obvious key question is how would this occur? Recently, we noted two bicupin proteins in the genome sequence of *S. sclerotiorum* (SS1G\_10796.1, 47% identical to *Ceriporiopsis subvermispora* Oxo1 [CsOxo1], and SS1G\_08814.1, 46% identical to CsOxo2). It has been suggested that cupins function as oxalate oxidases in *Ceriporiopsis subvermispora* (9). Oxalate oxidase (also known as germin-like in plants) catalyzes the generation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) from oxalate. Although it has yet to be shown whether *S. sclerotiorum* bicupin proteins have oxalate oxidase activity, they could be associated with oxalate-mediated ROS generation. Previous work in the Dickman lab has shown that oxalate-induced H<sub>2</sub>O<sub>2</sub> in plant tissue is involved in programmed cell death (17). This observation suggests that fungally produced OA is associated with ROS generation in plants. While mechanistic details are as yet unknown, our observations and previous studies show a consistent link between ROS generation and the production of OA.

There are several potential routes for oxalate biosynthesis. In *Sclerotinium rolfsii*, the oxidation of glyoxylate by glyoxylate dehydrogenase was suggested as a major pathway for oxalate biosynthesis (1). In addition, exogenous application of <sup>14</sup>CO<sub>2</sub> in *Aspergillus niger* showed that the biosynthesis of oxalate occurs by hydrolysis of oxaloacetate (18). Hammel and col-

leagues (13a) also provided evidence for oxalate production via oxidation of glycolaldehyde by the *Phanerochaete chrysosporium* glyoxal oxidase. Several cofactors, such as NAD<sup>+</sup>, are necessary to maintain these pathways. For example, the tricarboxylic acid (TCA) cycle, considered one of the main pools for the production of secondary metabolites, including oxalate and glyoxylate, has three rate-limiting enzymes dependent on NAD<sup>+</sup> (24), and glyoxylate dehydrogenase also uses one NAD<sup>+</sup> molecule to produce one oxalate molecule. Therefore, recycling of cofactors for a steady-state supply of NAD<sup>+</sup> and consumption of NADH could be required to produce oxalate. Transhydrogenase, which converts NADP<sup>+</sup> plus NADH to NADPH plus NAD<sup>+</sup>, can contribute to this recycling. For example, NADP<sup>+</sup> from NADPH oxidase and NADH from the TCA cycle and oxidation of glyoxylate can be transformed into NADPH and NAD<sup>+</sup>, respectively, by transhydrogenase, thereby providing cofactors for ROS generation and oxalate production. Hence, conversion of NADPH to NADP by NADPH oxidase may impact production of secondary metabolites, as noted by Lalucque and Silar (20), and transhydrogenase may contribute to this process. In line with this, *S. sclerotiorum* has a putative transhydrogenase (SS1G\_09058.1) which has the characteristic NAD(P) transhydrogenase beta subunit. Thus, this transhydrogenase may play a role in the generation of NADPH and NAD<sup>+</sup> from NADP<sup>+</sup> and NADH, respectively. Interestingly, transhydrogenase homologs have not been found in available genomes of the hemiascomycete yeasts and other unicellular fungi, from which *nox* genes are also absent (36). Moreover, the filamentous fungi for which NADPH oxidases have been reported and genome sequences are known also contain transhydrogenase homologs. This possibility, however, at this point is purely speculative and will require further experimentation.

In summary, prevention of NADPH oxidase activity by inactivation of *Ssnx1* or chemical inhibition of SsNox1 resulted in reduced oxalate levels that correlated with limited virulence. Notably, oxalate supplementation restored the virulence of *Ssnx1* silenced mutants to nearly wild-type levels. These results strongly suggest a novel and functional relationship between NADPH oxidase/ROS and the production of oxalate, both of which impact fungal pathogenicity. We have shown that OA can suppress the host oxidative burst (4). The decrease in oxalate production leads to strongly localized ROS production in the host plant (41). Mechanistic studies regarding how SsNox1 regulates oxalate production will give valuable insight into how fungal NADPH oxidases contribute to the pathogenic success of *S. sclerotiorum* and quite possibly other fungi.

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