Random T-DNA Mutagenesis Identifies a Cu/Zn Superoxide Dismutase Gene as a Virulence Factor of *Sclerotinia sclerotiorum*

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Agrobacterium-mediated transformation (AMT) was used to identify potential virulence factors in *Sclerotinia sclerotiorum*. Screening AMT transformants identified two mutants showing significantly reduced virulence. The mutants showed growth rate, sclerotial formation, and oxalate production similar to that of the wild type. The mutation was due to a single T-DNA insertion at 212 bp downstream of the Cu/Zn superoxide dismutase (SOD) gene (*SsSOD1*, SS1G_00699). Expression levels of *SsSOD1* were significantly increased under oxidative stresses or during plant infection in the wild-type strain but could not be detected in the mutant. *SsSOD1* functionally complemented the Cu/Zn SOD gene in a $\Delta sod1$ *Saccharomyces cerevisiae* mutant. The SOD mutant had increased sensitivity to heavy metal toxicity and oxidative stress in culture and reduced ability to detoxify superoxide in infected leaves. The mutant also had reduced expression levels of other known pathogenicity genes such as endo-polygalacturanases *spg1* and *spg3*. The functions of *SsSOD1* were further confirmed by *SsSOD1*-deletion mutation. Like the AMT insertion mutant, the *SsSOD1*-deletion mutant exhibited normal growth rate, sclerotial formation, oxalate production, increased sensitivity to metal and oxidative stress, and reduced virulence. These results suggest that *SsSOD1*, while not being required for saprophytic growth and completion of the life cycle, plays critical roles in detoxification of reactive oxygen species during host–pathogen interactions and is an important virulence factor of *Sclerotinia sclerotiorum*.

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic and non-host-specific fungal pathogen capable of infecting more than 400 plant species worldwide and can cause significant yield losses in many economically important crops, including pea, chickpea, soybean, sunflower, common bean, and canola valued at \$200 million per year in the United States alone (Boland and Hall 1994; Bolton et al. 2006; Hegedus and Rimmer 2005). The disease is difficult to manage due to its wide

host range, persistent survival structure (sclerotia), and lack of adequate host resistance (Bolton et al. 2006). Knowledge of pathogenic mechanisms of *S. sclerotiorum* is helpful for development of more effective disease-control strategies.

At present, many genes related to pathogenicity of *S. sclerotiorum* have been reported (Erental et al. 2007; Yajima et al. 2009). For example, Rollins (Rollins 2003; Rollins and Jurick 2007) used direct gene disruption to show that two genes, *pac1*, a pH-regulatory gene, and *sac1*, a single-copy adenylate cyclase gene, play key roles in pathogenesis of *S. sclerotiorum*. Kim and associates (2011) used RNA interference to demonstrate that *SsNox1*, one of the two NADPH oxidases (Nox) presumably involved in reactive oxygen species (ROS) generation, is required for virulence and pathogenic development of *S. sclerotiorum*. However, despite extensive studies on this pathogen, its pathogenic mechanisms are still incompletely understood because the pathogenesis of this fungus is very complex (Amselem et al. 2011; Hegedus and Rimmer 2005; Williams et al. 2011).

Agrobacterium-mediated transformation (AMT) has been shown to be an efficient tool for insertional mutagenesis and elucidation of gene functions in fungi (Giesbert et al. 2012; Michielse et al. 2005). Since first used in transformation of *Saccharomyces cerevisiae* (Bundock et al. 1995) in 1995, AMT has been adopted to transform a number of fungi to produce insertional mutation libraries and identify pathogenicity-related genes of *Botrytis cinerea* (Giesbert et al. 2012; Rolland et al. 2003), *Magnaporthe oryzae* (Betts et al. 2007; Choi et al. 2007; Meng et al. 2007), *Fusarium oxysporum* (Mullins et al. 2001), *Cryptococcus neoformans* (Idnurm et al. 2004), and *Leptosphaeria maculans* (Blaise et al. 2007). Meanwhile, AMT has been used to successfully transform *Sclerotinia sclerotiorum* using either ascospores or mycelia (Liberti et al. 2007; Weld et al. 2006). Therefore, AMT should be a useful tool for identification of additional potential virulence or pathogenicity factors in *S. sclerotiorum*.

The occurrence of ROS is one of the earliest cellular responses following successful pathogen recognition (Torres et al. 2006). The critical roles of ROS, including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($OH^\cdot$) produced by either fungal pathogens or their hosts in pathogenesis and defense-related activities, have been well established in numerous pathosystems (Heller and Tudzynski 2011; Kim et al. 2009; Kim et al. 2011; Li et al. 2011; Missall et al. 2004; Williams et al. 2011). The oxidative stress produced by ROS can react with and damage complex cellular molecules such as DNA, proteins, and lipids (Apel and Hirt 2004). To cope with the oxidative stress encountered during infection,

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pathogens must activate their antioxidant defenses. Superoxide dismutases (SOD) are metalloenzymes that form a primary cellular antioxidant defense system by detoxifying oxygen radicals via conversion of superoxide radicals to oxygen and H_2O_2 , which can then be removed by catalase and peroxidase (Vallino et al. 2009). There are some examples of SOD that contribute to virulence in phytopathogenic fungi (Rolke et al. 2004; Xie et al. 2010), including *S. sclerotiorum* (Veluchamy et al. 2012). Deletion of *bcsod1* caused retarded lesion development and proved that this gene is important for full virulence of *B. cinerea* (Rolke et al. 2004). In addition, increasing evidence shows that necrotrophic fungi, including *B. cinerea* and *M. oryzae*, themselves generate ROS and use it to colonize the host (Egan et al. 2007; Heller and Tudzynski 2011; Tenberge et al. 2002). During the infection process of *B. cinerea*, O_2^- accumulated in the fungal hyphal tips and H_2O_2 (either fungal or host origin) could be detected at the edge of the lesion, suggesting that the fungi might directly secrete H_2O_2 (a product of SOD) for the successful colonization (Tenberge et al. 2002).

In order to identify additional potential pathogenicity or virulence factors and to gain better understanding of the mechanisms of pathogenesis of *S. sclerotiorum*, we generated random transformants through AMT. Among a dozen or so transformants that showed reduced virulence, two were due to disruption of the *SsSOD1* gene by T-DNA insertion. This research was carried out to characterize one of the two *SsSOD1* mutants, test the roles of the SOD gene in virulence in protecting cell by detoxification of ROS, and confirm the function of *SsSOD1* employing yeast complementation tests. In addition, the functions of *SsSOD1* in *S. sclerotiorum* were further confirmed through gene replacement.

RESULTS

Transformation of *S. sclerotiorum* and screening for reduced virulence.

Mycelial fragments of *S. sclerotiorum* were transformed with *Agrobacterium tumefaciens* AGL-1, and more than 800 putative transformants were obtained. After four rounds of sequential hyphal tip purification on selective medium containing hygromycin B (HygB), all the transformants were screened for virulence phenotype on detached pea leaves. The area of disease lesion was recorded after 24, 48, and 72 h postinoculation (hpi). Transformant M388 showed significantly retarded disease development compared with the wild type (Fig. 1A).

The leaves inoculated with the wild type exhibited an average necrotic area of 3.1, 4.7, and 7.7 cm^2 at 24, 48, and 72 hpi, respectively. However, the average areas of necrosis for the mutant were 0.5, 1.5, and 3.0 cm^2 at 24, 48, and 72 hpi, respectively (Fig. 1B). In addition to the differences in lesion areas, the amount of mycelial growth was noticeably reduced on the pea leaves inoculated with the mutant M388 compared with the wild type at 48 hpi, based on the white, cottony mycelial mass characteristic of *S. sclerotiorum* infection (Fig. 1A). The results showed that the mutant M388 had significantly reduced virulence, suggesting that the gene or genes disrupted in the mutant play critical roles in the virulence of *S. sclerotiorum*.

Essentially no differences were detected in growth rate, sclerotial formation, and oxalic acid (OA) production between the mutant M388 and the wild-type strain. On potato dextrose agar (PDA), growth rate of the mutant M388 was slightly less than the wild type. At 72 hpi, the wild type can cover the whole plate (diameter of 8.5 cm). By contrast, the colony diameter of mutant M388 is approximately 8 cm. Mutant M388 showed no obvious differences in sclerotial formation on PDA plates compared with the wild type (Fig. 2). In addition, the intensity of yellow color of the pH-indicating medium amended with bromophenol blue showed no difference between the mutant M388 and the wild-type strain (data not shown). OA is an important pathogenicity factor for *S. sclerotiorum* (Cessna et al. 2000). Quantitative determinations of OA production in vitro were performed using high-performance liquid chromatography (HPLC). The wild type and the mutant M388 produced essentially the same amount of OA in culture (OA at 62.2 and 60.5 mg/g dry weight of mycelia, respectively) (Fig. 2).

Determination of T-DNA insertion number and genomic location and sequence characterization of the disrupted gene.

T-DNA insertion number in the transformants was determined using Southern blot analysis of *XhoI*-digested genomic DNA using the *HygB* gene as a probe. Southern blot experiments at high stringency showed single hybridization bands in most transformants, including mutant M388, but no band was detected in the wild type. In addition, the band sizes were different among transformants, indicating that the T-DNA insertion was random and the mutation in mutant M388 was due to a single T-DNA insertion (Supplementary Fig. S1).

The genomic location of the T-DNA insertion in mutant M388 was determined using inverse polymerase chain reaction (PCR). The inverse PCR designed for amplifying the left border

Fig. 1. Comparison of virulence between the wild type (WT) and transformant. **A**, Colonization of pea leaves inoculated with either the WT or the T-DNA insertion superoxide dismutase (SOD) mutant M388 of *Sclerotinia sclerotiorum* during the first 72 h of detached leaf assay. **B**, Quantification of disease lesion areas of necrosis on pea leaves inoculated with the WT or the mutant M388 of *S. sclerotiorum* ($N = 3$).

region of T-DNA amplified one 1,000-bp band from genomic DNA of the transformant M388. The complete sequence of this inverse PCR product was used as query to BLAST search the *S. sclerotiorum* genome sequence, which identified an 802-bp region in the genome of *S. sclerotiorum*. The T-DNA insertion site in the mutant M388 was at 212 bp downstream of the stop codon of Cu/Zn SOD (SS1G_00699) of *S. sclerotiorum* (termed *SsSOD1*).

The *SsSOD1* gene contains a 908-bp open reading frame (ORF), including four introns of 272, 67, 52, and 60 bp, respectively, which was confirmed by PCR analyses using the genomic DNA and cDNA as templates (data not shown). The ORF has the coding potential for a protein of 155 amino acids with a molecular weight of 15.9 kDa and an isoelectric point of 6.1. When compared with sequences in the GenBank, the deduced amino acid sequence was found to belong to the Cu/Zn SOD superfamily, suggesting that it can bind to Cu and Zn ions. Sequence analysis using SignalP 3.0 found no signal peptide in the N terminal of the protein.

Replacement of *SsSOD1*.

Because the insertion site of T-DNA in mutant M388 was located at the 3'-flanking region of *SsSOD1*, further analyses through a direct *SsSOD1* replacement in the wild type were performed to confirm the gene function. Two fragments containing the 5' flank and 3' flank of *SsSOD1* and *HygB*-split

marker were generated through double-joint PCR (Yu et al. 2004) and used to transform protoplasts of the wild-type strain (Supplementary Fig. S2). After four rounds of hyphal tip purification on selective medium, 12 *HygB*-resistant transformants were selected and tested for homologous integration by diagnostic PCR. PCR results using the two primer pairs KO5F/KO6R and KO1F/HY-R (Table 1) indicated that the *SsSOD1* gene was deleted in one of the transformants (data not shown), which was used in further analysis by Southern blot. Southern analysis using the 5'-flank sequence as a probe showed the different-sized bands between the wild type and the *SsSOD1*-deletion transformant. When the *SOD1* ORF sequence was used as a probe, the expected-size band was present only in the wild-type strain but absent in the *SsSOD1*-deletion mutant, indicating that the transformant is homokaryotic in *SsSOD1* deletion.

Similar to the AMT mutant M388, the *SsSOD1*-deletion mutant showed the normal growth rate on PDA, produced the same amount of OA in culture filtrate (OA at 58.7 mg per gram of mycelia dry weight), and showed no detectable change on sclerotial formation as compared with the wild-type strain (Fig. 2). To ascertain the virulence phenotype of the *SsSOD1*-deletion mutant, inoculations were performed on leaves of intact pea and bean plants (Supplementary Fig. S3). At 72 hpi, the leaves inoculated with the wild type collapsed. In contrast, the leaves inoculated with the *SsSOD1*-deletion

Fig. 2. Sclerotial formation and oxalate production of *Sclerotinia sclerotiorum* strains. **A**, Sclerotial formation of *S. sclerotiorum* strains on potato dextrose agar 2 weeks postinoculation. **B**, Oxalate production of the wild-type strain, *Agrobacterium*-mediated transformation mutant M388, and *SsSOD1*-deletion mutant grown on potato dextrose broth at 7 days postinoculation. Data were averages of three replicates.

mutant stood up. The average lesion area of leaves infected by the *SsSOD1*-deletion mutant is significantly reduced compared with the wild type, indicating that *SsSOD1* deletion had the same effect as T-DNA insertion in the 3'-end untranslated region (UTR) of the *SsSOD1* gene found in mutant M388.

Functional analysis of *SsSOD1*.

The potential functions of the *SsSOD1* gene in *S. sclerotiorum* were investigated in three ways: regulation of the *SsSOD1* expression by oxidative stress condition, tolerance to metal toxicity and oxidative stress, and heterologous complementation of SOD in a yeast mutant.

To test the effect of oxidative stress on *SsSOD1* expression, mycelia of the wild type were treated with 0.5 and 2.0 mM H_2O_2 . Quantitative PCR showed that expression of *SsSOD1* was significantly increased in the culture treated with 0.5 mM H_2O_2 from 0.5 to 2 h post-treatment (hpt) (Fig. 3). Furthermore, a 48-fold increase of *SsSOD1* transcript over control (0 hpt) was detected a half-hour after application of 2 mM H_2O_2 ; however, this dramatic increase was transient and its expression levels gradually decreased thereafter (Fig. 3). The results demonstrate that expression of *SsSOD1* is regulated by and responsive to oxidative stress.

Tolerance to Zn toxicity in the mutant M388 and the *SsSOD1*-deletion mutant was similar compared with that of the wild type in PDA amended with Zn. The Zn toxicity was obvious by comparing the growth rates in plain PDA and in PDA amended with Zn. Mycelial growth of both the wild-type and transformant strains at 5 mM $ZnSO_4$ was reduced compared with that on PDA without addition of Zn (Fig. 4). The wild-type strain and an ectopic transformant M322 that had a T-DNA insertion at a different but undetermined location were able to grow at 5 mM $ZnSO_4$, albeit severely limited, whereas the growth of the mutant M388 and *SsSOD1*-deletion mutant was more severely inhibited, showing that mutation at the *SsSOD1* gene significantly impaired tolerance to Zn toxicity. However, the AMT mutant M388 and *SsSOD1*-deletion mutant were still viable because they were able to start normal growth when transferred to plain PDA. Tolerance to oxidative stress of

the mutant M388 and the *SsSOD1*-deletion mutant was compared with that of the wild type in PDA amended with *N,N'*-dimethyl-4,4'-bipyridinium dichloride (paraquat). Similarly, paraquat at 5 mM completely inhibited the growth of the mutant M388 and *SsSOD1*-deletion mutant but had no noticeable effect on the growth of the wild type and the ectopic transformant M322 (Fig. 4), suggesting that disruption of the *SsSOD1* gene had reduced ability to tolerate oxidative stress. The mycelium of the mutants on the inoculation plugs after 48 h of incubation on paraquat-amended PDA was not viable because they failed to grow after transferring to plain PDA.

For the heterologous complementation tests, full-length cDNA of the *SsSOD1* was cloned into the yeast expression vector p415ADH. The constructs containing the *SsSOD1* (p415ADH-*SsSOD1*) or the empty vector (p415ADH) were transformed into a *Saccharomyces cerevisiae* mutant defective in

Fig. 3. Expression of *SsSOD1* relative to β -tubulin gene expression induced by oxidative stress. Mycelia of the wild type of *Sclerotinia sclerotiorum* treated with H_2O_2 at the indicated concentrations and incubation times. Boxes and bars represent averages and standard errors, respectively, of three independent biological replicates.

Table 1. Polymerase chain reaction primers used in this study^a

Primer	Sequence (5'-3')	Usage
Hph-F	GAGCCTGACCTATTGCATCTC	Hygromycin B gene
Hph-R	CCGTCAACCAAGCTCTGATAG	...
LB5IP	AGTCGTTTACCCAGAATGCACAGGTACACT	Inverse PCR
RB5IP	CTTGACACAAATTGGATGTCCATCTTCGAAAC	...
β tubF	TTGGATTGTGCTCTTTGACCAG	β -tubulin transcripts
β tubR	AGCGGCCATCATGTCTTAGG	...
sodF	CCACTGGTTCCGTGAAGA	<i>SsSOD1</i> transcripts
sodR	ACCACAAGCTGGTCTGGTTC	...
sspg1F	GTCTCCGGCGTTACCTACAA	<i>Sspg1</i> transcripts
sspg1R	TAGCGGAGGAAGAGACGGTA	...
sspg3F	ACAAACGGCGTTACCATCTC	<i>Sspg3</i> transcripts
sspg3R	ATGGGAAGTTGCAGGA	...
SOD-BamHI	CGGGATCCATGGTTAAAGCAGTTGCTACCGT	Construction of p415ADH- <i>SsSOD1</i>
SOD-HindIII	CCAAAGCTTTTATGCAGCAATACCAA TAACACC	...
KO1F	TAGTACAGGGTTGTGAGGCAAT	<i>SsSOD1</i> gene replacement
KO2R	TTGACCTCCACTAGCTCCAGCCAAGCCTATCGATGTCTTCTCCGTTTTGT	...
KO3F	GAATAGAGTAGATGCCGACCGGGTTACTAGATTGAACCGAATCACGAA	<i>SsSOD1</i> gene replacement
KO4R	AAGTAGGATGTGCGAAATTGAAA	...
KO5F	CCACTGGTTCCGTGAAGA	<i>SsSOD1</i> gene replacement
KO6R	ACCACAAGCTGGTCTGGTTC	...
KO7F	CCGTCCTTACAACTATGAGCA	<i>SsSOD1</i> gene replacement
KO8R	TTCGCACATCTACTTATTTTCG	...
HYG-F	GGCTTGGCTGGAGCTAGTGGAGGTCAA	<i>SsSOD1</i> gene replacement
HYG-R	AACCCGCGGTGCGCATCTACTCTATTC	...
YG-F	GATGTAGGAGGGCGTGGATATGTCCT	<i>SsSOD1</i> gene replacement
HY-R	GTATTGACCGATTCTTTCGCGTCCGAA	...

^a PCR = polymerase chain reaction and SOD = superoxide dismutase.

the Cu/Zn SOD gene (MAT α gal1 trp1-1 his leu2-3,-112 ura3-50 sod1D::TRP1). The yeast transformants carrying p415ADH vector or p415ADH-*SsSOD1* construct were plated onto synthetic dextrose (SD) agar (minus leucine) plates with or without Cu or Cd (Fig. 5). The transformants carrying p415ADH vector grew only in the medium without the heavy metals and cannot grow in the media containing heavy metals (Fig. 5). In contrast, the yeast transformants carrying the p415ADH-*SsSOD1* construct were able to grow and developed colonies under the heavy metal stress (Fig. 5). The results indicate that the *SsSOD1* gene of *Sclerotinia sclerotiorum* can functionally complement the *SOD1* gene of *Saccharomyces cerevisiae*.

Transcriptional analyses of *SsSOD1* and polygalacturonase genes.

Transcription levels of the *SsSOD1* gene were monitored in the wild type and the mutant in vivo. Transcription levels of *SsSOD1* of the wild type significantly increased over time upon inoculation onto pea leaves (Fig. 6). However, *SsSOD1* transcript could not be detected in mutant M388 during infection of pea plants or in vitro cultures, suggesting that insertion at the 3' UTR of the *SsSOD1* gene prevented its expression in the transformant M388 and rendered the gene nonfunctional.

OA and polygalacturonases (PG) are important virulence factors of *Sclerotinia sclerotiorum* (Rollins 2003). Because the mutation at *SsSOD1* did not affect OA production (Fig. 2), experiments were carried to test whether mutation at the *SsSOD1* gene affects expression of PG genes during plant infection. The expression of two endo-PG genes, *spg1* and *spg3*, in the wild type and mutant were analyzed by real-time reverse-transcription (RT)-PCR (Fig. 7). The level of *spg1* transcript in the wild-type strain was induced at 24 to 72 hpi. In contrast, in the

mutant, it reached a higher level (9.2-fold over that in the wild type at 24 hpi); however, this increase was transient and then decreased to levels lower than those in the wild type at 48 to 72 hpi (Fig. 7A). The levels of *spg3* transcript in the wild-type strain were stable (less than twofold change) between 24 and 72 hpi. However, its expression levels were significantly and consistently lower in the mutant than in the wild-type strain at 24 to 72 hpi (Fig. 7B). The results showed that the mutation of *SOD1* gene had obvious negative effects either directly or indirectly on expression of PG genes, especially on *spg3*.

Increased accumulation of superoxide in disease lesions infected by SOD mutants.

An important function of SOD is to convert superoxide into lesser toxic H₂O₂ and oxygen. Disease lesions caused by the SOD mutant should show more accumulated superoxide relative to those of the wild type. Leaves infected with the wild-type and mutant strains were stained with nitroblue tetrazolium chloride (NBT) to detect production and accumulation of superoxide. The results showed that more superoxide accumulated in pea leaves inoculated with the AMT mutant M388 and the *SsSOD1*-deletion mutant than in leaves inoculated with the wild type at 72 hpi. In the leaves infected with the wild type, the accumulation of superoxide was low in intensity of staining and only found in the margins of the lesions. In contrast, it occurred in the whole lesion areas in the leaves infected with either the mutant M388 or the *SsSOD1*-deletion mutant (Fig. 8).

DISCUSSION

AMT is a powerful tool for studying pathogenicity genes of fungi because it does not require prior knowledge of the genes

Fig. 4. Sensitivity of *Sclerotinia sclerotiorum* strains to Zn toxicity and oxidative stress. The wild-type (a), ectopic transformant M322 (b), *Agrobacterium*-mediated transformation mutant M388 (c), and *SsSOD1*-deletion mutant (d) were grown for 2 days on potato dextrose agar containing Zn or *N,N'*-dimethyl-4,4'-bipyridinium dichloride (paraquat) at indicated concentrations.

or preparation of protoplasts (Choi et al. 2007; Giesbert et al. 2012; Kemppainen et al. 2008; Michiels et al. 2005; Münch et al. 2011; White and Chen 2006). Another transformant, M196, was generated in a different transformation experiment from M388 but both had T-DNA insertions at exactly the same nucleotide locations, possibly due to microsequence homology to the left terminus of T-DNA, as reported in *M. oryzae* (Meng et al. 2007). Although T-DNA insertions were proven to be random across the entire genome, studies showed T-DNA insertions preferentially insert toward promoters and 3' UTR (Choi et al. 2007; Giesbert et al. 2012; Münch et al. 2011; Wang 2008). Our results showed that the T-DNA insertion at the 3' UTR prevented expression of *SsSOD1*, suggesting that the integrity of the sequence downstream of the insertion site is essential for *SsSOD1* expression.

Cu/Zn SOD are usually located in cytosol and function to protect the eukaryotic cells against endogenous superoxide production (Holdom et al. 1996). *SsSOD1* contains no signal peptide, a typical feature of cytosolic enzymes. Two lines of evidence were obtained to demonstrate the functions of *SsSOD1*. First, mutation at this gene increased sensitivity of *S. sclerotiorum* to Zn toxicity and oxidative stress. Second, the *SsSOD1* gene can restore or increase tolerance of the *SOD*-defective mutant of *Saccharomyces cerevisiae* to heavy metal toxicity, functionally complementing the *SOD* gene in yeast.

Fig. 5. Yeast complementation assay of *SsSOD1*. Heterologous complementation tests showing increased tolerance to oxidative stress conferred by *SsSOD1* in a yeast mutant defective of Cu/Zn superoxide dismutase (*SOD*). Yeast mutants harboring the expression vector (p415ADH) alone or the expression vector containing the *SsSOD1* gene (p415ADH-*SsSOD1*) were grown on synthetic dextrose (minus leucine) agar plates with CuSO_4 or CdSO_4 at indicated concentrations.

Cu/Zn SOD, apparently a single-copy gene in most fungal genomes, has been intensively studied in a number of fungi (Chary et al. 1994; Moore et al. 2002; Rolke et al. 2004). A general consensus is that Cu/Zn SOD increases tolerance to oxidative stress, while not being required for saprophytic growth or viability of pathogenic fungi. For example, in the human pathogen *C. neoformans* var. *gattii*, an *SOD1*-deletion mutant was extremely sensitive to menadione (a superoxide-generating agent) and had severely attenuated virulence on mice but had no difference on mating, sporulation, growth, and stationary phase survival from the wild type (Narasipura et al. 2003). Mutants with *SOD1* deletion of *B. cinerea* maintained normal growth and sporulation but showed increased sensitivity to the H_2O_2 -generating compound paraquat (Rolke et al. 2004), whereas their virulence phenotypes varied from retarded lesion development (attenuated virulence) to no difference from the wild type, depending on assay conditions (Patel et al. 2008; Rolke et al. 2004). In the biotrophic pathogen *Claviceps purpurea*, *SOD1* is nonessential not only for normal growth but also for tolerance to oxidative stress and virulence (Moore et al. 2002). A proposed explanation for such nonessentiality of *SOD1* under low-stress conditions is that other existing detoxifying systems can compensate for the lost *SOD1* activity, which is supported by the facts of upregulated mitochondrion-located MnSOD in Cu/Zn SOD-deletion mutants of *Neurospora crassa* (Chary et al. 1994) and enhanced production of mannitol (which can function as a scavenger of ROS) in the *SOD1*-deletion mutant of *C. neoformans* var. *gattii* (Narasipura et al. 2003). In our study, the AMT SOD mutant and the *SsSOD1*-deletion mutant had normal growth, normal sclerotial formation, and oxalate production equal to the wild type, suggesting that *SOD1* is not required for saprophytic growth of *Sclerotinia sclerotiorum*. The fact that both *SOD1*-insertion and -deletion mutants still caused appreciable disease, albeit reduced virulence, in both detached-leaf and in planta assays, further supports the conclusion that *SOD1* is not essential for *S. sclerotiorum* under low-stress conditions. However, in a recent study, Veluchamy and associates (2012) reported that *SOD1*-deletion mutants of *S. sclerotiorum* lost the ability to form sclerotia and had reduced production of oxalate, in addition to increased sensitivity to oxidative stress and reduced

Fig. 6. Expression of *SsSOD1* during infection of pea leaves. Detached pea leaves were inoculated with an agar plug (3 mm in diameter) containing actively growing mycelia of either the wild type or the transformant M388. Values were relative to β -tubulin gene expression in RNAs isolated from disease lesions 24, 48, and 72 h postinoculation. Boxes and bars represent averages and standard errors, respectively, of three independent biological replicates.

virulence. The loss of sclerotial formation would suggest a serious disruption of the life cycle by the *SOD1* deletion, contrary to general beliefs of *SOD1*'s roles in pathogenic fungi (see discussion above). We could not offer any explanation for such different phenotypes of *SOD1*-deletion mutants between the study of Veluchamy and associates (2012) and this study, other than to point out that this potentially could be due to different strains of *S. sclerotiorum* being used in the studies that may have different abilities to compensate for the lost activity of *SsSOD1* in the mutants.

While being nonessential for growth under low-stress conditions, SOD is known to contribute to fitness under high oxidative stress and to virulence. Because oxidative burst is a general characteristic of plant defense induction upon recognition of pathogen attack (Heller and Tudzynski 2011), SOD is required

for full virulence. In establishing infection and throughout colonization of host tissue, the growing front of the disease lesion encounters high levels of ROS, as shown by the accumulated superoxide in the lesion margins of wild-type-infected leaves (Fig. 8). SOD may contribute to virulence in several ways. First, SOD activities can detoxify or neutralize dangerous levels of superoxide either produced endogenously or presented by the host in resistance reactions. Without SOD, toxic levels of superoxide will accumulate in and around mycelium in infected plants (Fig. 8). The accumulated ROS will weaken the mycelium and render the fungus incapable of launching a full attack on the host, resulting in reduced virulence. Although *SsSOD1* does not possess a signal peptide, *SsSOD1* is the only copy of Cu/Zn SOD found in the *S. sclerotiorum* genome and is a small protein (155 amino acids). Small proteins may get secreted

Fig. 7. Expression levels of polygalacturonase genes. Expression levels of **A**, endo-polygalacturonase 1d (*spg1*) and **B**, endo-polygalacturonase 3 (*spg3*) relative to β -tubulin gene expression during infection of pea leaves. Detached leaves were inoculated with an agar plug (3 mm in diameter) containing actively growing mycelia of either the wild type or the transformant M388. Lesions were excised from leaves 24, 48, and 72 h postinoculation for RNA isolation. Boxes and bars represent averages and standard errors, respectively, of three independent biological replicates.

Fig. 8. Accumulation of superoxide in disease lesions. Detection of superoxide accumulated in pea leaves inoculated with the wild-type strain, *Agrobacterium*-mediated transformation mutant M388, and *SsSOD1*-deletion mutant at 72 h postinoculation before (top) and after (bottom) staining with nitroblue tetrazolium chloride.

through alternative secretion mechanisms (Lum and Min 2011; Nickel 2003). SOD without signal peptide in other fungi were found in culture filtrates, suggesting that they are secreted (Chen et al. 1997; Hamilton and Holdom 1997; Holdom et al. 1996). It is possible that the *SsSOD1* in *S. sclerotiorum* is also secreted even without signal peptide. Second, SOD carries out the first enzymatic reaction of the ROS detoxification process. The subsequent products of the detoxification process include H_2O_2 and OH^- . These subsequent products could be required at sufficient levels for successful *S. sclerotiorum* attack on the host plant. Nullification of SOD would diminish supply of these subsequent products and, consequently, reduce *S. sclerotiorum* virulence. This explanation is also consistent with the fact that silencing *SsNox1* (a NADPH-dependent superoxide-generating enzyme) also reduced virulence (Kim et al. 2011). Third, inactivation of SOD may have pleiotropic effects that include reduced expression of other virulence factors. Deletion of *SOD1* in *C. neoformans* var. *gattii* resulted in defects in expression of a number of virulence factors such as laccase, urease, and phospholipase (Narasipura et al. 2003). Virulence factors of *S. sclerotiorum* include OA, PG, and a suite of other cell-wall-degrading enzymes (Rollins 2003). Although oxalate production was not affected by the *SsSOD1* mutations, expression of two PG (*sspg1* and *sspg3*) was significantly reduced (Fig. 7). Considering the initial dramatic but transient increase of *sspg1* expression in the SOD mutant upon inoculation onto pea leaves (Fig. 7), the effect of SOD mutation on expression of PG was probably indirect due to the weakened state of the mycelium under ROS stress. Veluchamy and associates (2012) also reported reduced virulence in *SsSOD1*-deletion mutants and attributed it to reduced oxalate production because their mutants produced only half the amount of oxalate as the wild type (Veluchamy et al. 2012; Williams et al. 2011). However, we cannot attribute the reduced virulence of the *SOD* mutants in this study to reduced oxalate production because i) both the AMT SOD mutant and the *SsSOD1*-deletion mutant produced the same amount of oxalate as the wild type did (Fig. 2) and ii) a naturally occurring strain of *S. sclerotiorum* produced less than half the amount of oxalate as did its cohort strains but was just as virulent as the other strains (Attanayake et al. 2012).

In summary, we have shown that AMT is an efficient tool to identify pathogenicity or virulence factors in *S. sclerotiorum*. The SOD function of the *SsSOD1* gene was confirmed using heterologous functional complementation. Our results demonstrated that the *SsSOD1* gene, while not being required for saprophytic growth and completion of the life cycle, is necessary for tolerance to exogenous ROS and oxidative stress during host–pathogen interactions, and deletion or mutation of the *SsSOD1* gene significantly impairs virulence of *S. sclerotiorum*.

MATERIALS AND METHODS

Strains and culture conditions.

The *S. sclerotiorum* wild-type isolate (WMA1) collected from pea was maintained at 24°C on PDA. Long-term storage stock was maintained at –20°C as sclerotia and desiccated mycelia-colonized filter paper. Mycelia of *S. sclerotiorum* for transformation were grown in 50 ml of potato dextrose broth (PDB) on a rotary shaker (100 rpm) at 24°C for 4 days. *A. tumefaciens* AGL-1 containing plasmid pDJW5 (White and Chen 2006) was used for transformation and was grown in YEP (1% yeast, 1% peptone, 0.5% NaCl) (Lichtenstein and Draper 1986), minimal medium (MM), induction medium (IM) (Bundock et al. 1995), or Luria-Bertani (LB) medium, as appropriate, at 25 to 28°C. HygB-resistant fungal transformants were grown on a selective agar medium (SM) (PDA amended with hygromycin at 150 µg/ml). Mycelia for DNA and RNA isolations were

grown for 4 days in PDB or on PDA overlaid with cellophane membranes, and ground to a fine powder in liquid nitrogen.

The *Δsod1* *Saccharomyces cerevisiae* mutant (yeast haploid deletion mutant collection, Open Biosystems, DTY116-*ΔSOD1*, provided by D. J. Thiele, Duke University, Durham, NC) has the following genotype: MAT α gal1 trp1-1 his leu2-3,-112 ura3-50 *sod1Δ*::TRP1. The yeast strain was grown and maintained on rich YPD (1% yeast, 1% peptone, 2% glucose, 2% agar) medium.

Fungal transformation.

AMT of *Sclerotinia sclerotiorum* followed the methods of Liberti and associates (2007), with minor modifications. *A. tumefaciens* AGL-1 containing plasmid pDJW5 was grown for approximately 36 h at 28°C in LB containing kanamycin and rifamycin. Cultures were washed with MM, then IM, diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 to 0.2 with IM, and precultivated for 6 h at 28°C (Bundock et al. 1995). Following precultivation, bacterial cells were centrifuged at 3,000 rpm, washed once with fresh IM, and suspended in 1 volume of fresh IM. Mycelia for transformation were blended aseptically for 10 s in a Waring blender, harvested by centrifugation at 3,000 rpm for 10 min, and suspended in IM at 25 mg (wet weight)/ml prior to co-cultivation. Washed *Agrobacterium* cell suspensions were mixed with an equal volume of mycelia fragment suspensions, and 200-µl aliquots of the mixtures were plated onto prepared dialysis membrane (MWCO 12,000; Spectrapore, Rancho Dominguez, CA, U.S.A.) overlaid on co-cultivation-medium (IM agar containing acetosyringone at 200 µmol/liter); then, the co-cultivation plates were incubated in the dark for 3 days at room temperature. Following co-cultivation, the membranes containing the co-cultivate were transferred and inverted to SM containing chloramphenicol at 50 µmol/liter and cefotaxime at 200 µmol/liter. The membranes were removed after an additional 2 days of co-cultivation at room temperature. *S. sclerotiorum* transformants that arose on the SM 4 to 8 days after transfer were immediately moved to new SM. In order to obtain the purified transformants, four rounds of hyphal tip isolations on SM were routinely carried out under a dissection microscope (Zeiss stemi 2000-C; Oberkochen, Germany) to establish colonies that maintained rapid growth on SM.

Pathogenicity assay.

A detached-leaf assay was used to screen transformants for altered pathogenicity. Leaves were excised from 4-week-old plants of pea ('Guido') grown in 1-liter plastic pots containing LA4 Aggregate Plus Sunshine Professional Peat-Lite Mix (Sungro Horticulture, Vancouver, Canada) in a greenhouse under day and night temperatures of 25 and 18°C, respectively. Excised leaves were placed in 9-cm petri dishes lined with number 1 Whatman filter paper saturated with sterile water. Individual leaves were inoculated with single mycelial-colonized agar plugs (0.5 cm in diameter) obtained from the expanding colony margins on PDA of either the wild type or transformants. Inoculated leaves were maintained at near 100% relative humidity at room temperature.

Leaves were photographed between 24 and 72 hpi. The area of the necrotic lesion on each leaf was calculated by the analysis of images using the software Assess: Image Analysis Software for Plant Disease Quantification (American Phytopathological Society Press, St. Paul, MN, U.S.A.). The lesion areas were calibrated based on area of a square (1 cm²) paper that was included in each photograph. Each treatment (isolate or transformant) was performed with three leaves each time, and performed three times.

An intact plant assay was used to confirm the pathogenicity of the *SsSOD1*-deletion mutant. Leaves of 4-week-old plants

of pea (Guido) or bean (*Phaseolus vulgaris* L.) 'Great Northern Tara' were inoculated with single mycelium-colonized agar plugs (3 mm in diameter) of the wild type or mutants. The infected plants were incubated in a plastic propagator box at 25°C under natural illumination. At least six infections per strain were tested and documented photographically at 72 hpi.

Colony morphology, growth rate, and oxalate production.

Growth rates were determined by measuring colony diameters on PDA. Plates (three plates per isolate) were inoculated with an agar plug (3 mm in diameter) of mycelia from the edge of a 2-day-old PDA culture. Measurements were taken at 24, 48, and 72 h.

Oxalate production was measured in two ways: color change of a pH-indicating solid medium and oxalate accumulation in a liquid medium using HPLC detection. The pH-indicating medium consisted of PDA plates amended with bromophenol blue at 50 mg/liter, a concentration that did not affect growth rate of *S. sclerotiorum* (Attanayake et al. 2012). Production and secretion of OA by *S. sclerotiorum* changed the medium color from blue to yellow, and the intensity of the yellow color is a semiquantitative measure of OA production (Attanayake et al. 2012). Plates were inoculated in a manner as in the growth rate experiments and incubated at 22°C, and the color change of the plates was recorded at 48 h.

To determine OA accumulation in liquid medium, 25 ml of PDB in 250-ml flasks with three replicates per isolate were inoculated with four agar plugs (3 mm in diameter) of mycelia from the edge of 2-day old colonies of either the wild type or the mutants. Cultures were shaken at 100 rpm for 7 days at room temperature, then harvested by vacuum filtration through Whatman number 1 filter paper. Mycelium dry weight was determined and culture filtrates were analyzed for OA using HPLC. HPLC was performed using an Agilent 1100 series system with a Agilent Eclipse XBD-C18 column (4.6 by 250 mm) and ultraviolet detection at 210 nm as previously described (Attanayake et al. 2012). OA concentration was calculated with a standard curve. The growth rate and oxalate accumulation experiments were repeated three times.

RNA and DNA preparation.

DNA was extracted from mycelia of wild-type and mutant strains using the method of Rollins and Dickman (2001), and RNA was extracted from infected leaf tissues and mycelia of wild-type and mutant strains with TRIzol (Gibco BRL, Rockville, MD, U.S.A.) in accordance with the manufacturer's instructions. In addition, RNA was isolated from mycelia of the wild-type strain treated with 0.5 and 2 mM H₂O₂ for detection of *SsSOD1* gene expression levels using real time RT-PCR.

Molecular confirmation of transformation events.

To check for insertion of T-DNA in the genome of *S. sclerotiorum*, both PCR and Southern hybridization techniques were used, as previously described (White and Chen 2006). PCR was performed using primers hph-F and hph-R (Table 1) for detecting the presence of the *hygB* gene. The copy number and randomness of T-DNA integration were determined by Southern hybridization. Genomic DNA (10 µg) was digested with *EcoRI*, and the digested fragments were separated on a 0.8% agarose gel for 4 h, then transferred to a nylon membrane (Millipore, Billerica, MA, U.S.A.). A probe was generated by PCR using the hph-F and hph-R primers with the PCR digoxigenin (DIG) Probe Synthesis Kit (Roche, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions. Aqueous hybridizations were performed at 65°C overnight and detected using the DIG Luminescent Detection Kit (Roche) according to the manufacturer's instructions.

Inverse PCR for identifying DNA regions flanking T-DNA insertions.

To identify genomic DNA sequences flanking T-DNA insertions, inverse PCR was used (White and Chen 2006). To sequence the DNA region flanking the T-DNA left border, DNA was digested with *KpnI*. Aliquots of the digest were used in ligation reactions overnight at 10°C. PCR was performed using the ligation reactions as DNA templates using Elongase (Invitrogen, Carlsbad, CA, U.S.A.), with primers LB5IP and RB5IP (Table 1) for 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 3 min. Products were separated on 1% agarose gels (IBC BioExpress, Kaysville, UT, U.S.A.). Reactions yielding single products with the expected size (>460 bp of the T-DNA) were cloned into plasmid pGEM-T Easy vector and then sequenced using primer T7 or SP6 at the Washington State University Bioinformatics Core, Pullman.

Real-time RT-PCR.

Real-time RT-PCR assays were used to monitor the transcripts of interested genes. The interested genes include *SsSOD1* (SOD), *sspg1* (endo-PG 1d), and *sspg3* (endo-PG 3). The house-keeping *β-tubulin* gene was included as control for comparisons. Primers for *β-tubulin* amplification were *βtubF* and *βtubR* (Erental et al. 2007) (Table 1). The three pairs of primers for *SsSOD1* (*sodF/sodR*), *sspg1* (*sspg1F/sspg1R*), and *sspg3* (*sspg3F/sspg3R*) were designed by utilizing Primer3 software (Table 1).

First-strand cDNA synthesis was prepared from total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A.) according to manufacturer's instructions. Real-time RT-PCR analyses were performed using the Bio-Rad iQ5 Real-Time PCR Detection System. RT-PCR mixtures were composed of 12 pmol of each primer, 12.5 µl of SYBR Green Supermix (Bio-Rad), 2 µl of cDNA (a 1:5 dilution of the 20-µl cDNA product), and nuclease-free water to a final volume of 25 µl. The PCR conditions consisted of a denaturing step at 95°C for 2 min; a DNA denaturization step at 95°C for 10 s; both annealing and extension steps each at 58°C for 20 s for 40 cycles; and 65 to 95°C with a 0.5°C increment, each temperature for 5 s, to obtain the melting curve. In all experiments, samples were amplified in duplicate as technical replicates, and the average cycle threshold was then calculated and used to determine the relative expression of each gene. The quantification of the relative transcript levels was performed with the comparative cycle threshold method normalization (Livak and Schmittgen 2001). The transcript levels of target genes were normalized against the *β-tubulin* gene transcript levels. Each experiment was repeated three times in the same manner as biological replicates, and the averages of the three biological replicates and standard errors of the relative expression values were calculated.

Generation of *SOD1* gene replacement construct and mutant.

In order to confirm the gene function of *SsSOD1*, an *SsSOD1*-deletion mutant was generated using the split-marker gene replacement method (Li and Rollins 2010). The two transforming DNA fragments of the gene replacement constructs overlapping within the *hygB* gene were amplified using a double-joint PCR modified from the method previously described (Kim et al. 2005; Yu et al. 2004), each containing a flank of *SOD1* gene and *hygB*-split marker. In the first round of PCR, the flanks of *SsSOD1* were amplified from the wild-type *S. sclerotiorum* genomic DNA with primer pairs KO1F/KO2R and KO3F/KO4R and a 1.9-kb *hygB* cassette was amplified from pDWJ5 with primer pairs HYG-F/HYG-R (Table 1). The reverse primer KO2R (for the 5' flank) and the forward primer KO3F (for the

3' flank) carried 27-bp sequence tails that overlapped with the 5' and 3' ends of the *hygB* cassette, respectively. In the second round of PCR, each flank amplicon was fused to the *hygB* cassette through PCR splicing by overlap extension. The resulting PCR products were used as the template for the third round of PCR, with the new primer pairs KO7F/HY-R for the 5' construct and YG-F hy/KO8R for the 3' construct (Table 1). The PCR products (5' construct and 3' construct) were transformed into protoplasts of the wild-type strain as described by Rollins (2003). Fungal transformants carrying the *hygB* gene were selected on regeneration medium containing HygB. Putative *SsSOD1*-deletion mutants were screened by PCR with primers KO5F/KO6R and KO1F/HY-R (Table 1) and further confirmed by Southern blot analyses, with the *SOD1* gene and its upstream flanking sequence as probes.

Tests of tolerance levels to Zn toxicity and oxidative stress.

For tests of tolerance to Zn toxicity and oxidative stress, the wild type, an ectopic transformant, and mutants were grown for 4 days on PDA with or without the stress agents ZnSO₄ and paraquat (Sigma-Aldrich, St. Louis). At the conclusion of the experiment, agar plugs of the mutants on the plates with stress agents were transferred to fresh PDA to check for viability.

Yeast complementation assays.

The full-length *SsSOD1* cDNA sequence was first amplified under standard PCR conditions using the primers (SOD-BamHI/SOD-HindIII) containing respective restriction enzyme sites (Table 1). The resulting product was digested with *Bam*HI and *Hind*III and cloned into the yeast expression vector p415ADH that had been digested previously with *Bam*HI and *Hind*III. The p415ADH-*SsSOD1* construct or the empty p415ADH vector were then transformed (Rose et al. 1990) into chemically competent cells of *S. cerevisiae* DTY116 (MAT α gal1 trp1-1 his leu2-3,-112 ura3-50 sod1D::TRP1) (Labbe et al. 1999). Transformants were grown at 30°C for 2 to 3 days on selective (minus leucine) SD agar medium and then inoculated in liquid (minus leucine) SD medium. When the cells reached an OD₆₀₀ of 0.3, they were plated in triplicate on SD agar plates with or without 100 μ M CuSO₄ or 10 μ M CdSO₄.

Histochemical detection of superoxide.

Detection of superoxide accumulation in the infected leaves was performed by staining with NBT (Grellet Bournonville and Díaz-Ricci 2010). Detached pea leaves inoculated with the wild type and transformants at 72 hpi were immersed in 100 ml of potassium phosphate buffer (50 mM; pH 7.8) containing 0.1% NBT and 10 mM sodium azide. Leaves were vacuum infiltrated as described above for 2 min, incubated in the dark for 2 h and then in 96% (vol/vol) ethanol to completely remove chlorophyll. Photographs were taken before and after staining to facilitate locating superoxide accumulation relative to lesion area.

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