Sclerotinia sclerotiorum Thioredoxin Reductase Is Required for Oxidative Stress Tolerance, Virulence, and Sclerotial Development

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Sclerotinia sclerotiorum is a destructive ascomycete plant pathogen with worldwide distribution. Extensive research on different aspects of this pathogen’s capability to cause disease will help to uncover clues about new ways to safely control Sclerotinia diseases. The thioredoxin (Trx) system consists of Trx and thioredoxin reductase (TrxR), which play critical roles in maintenance of cellular redox homeostasis. In this study, we functionally characterized a gene encoding a TrxR (SsTrr1) in S. sclerotiorum. The amino acids of SsTrr1 exhibited high similarity with reported TrxRs in plant pathogens and targeted silencing of SsTrr1 lead to a decrease in TrxR activities of mycelium. SsTrr1 showed high expression levels during hyphae growth, and the levels decreased at the different stages of sclerotial development. SsTrr1 gene-silenced strains produced a smaller number of larger sclerotia on potato dextrose agar medium. The observations were consistent with the inhibitory effects on sclerotial development by the TrxR inhibitor, anrunofin. The expression of SsTrr1 showed a dramatic increase under the oxidative stress and the hyphal growth of gene-silenced strains showed more sensitivity to H₂O₂. SsTrr1 gene-silenced strains also showed impaired virulence in different hosts. Taken together, our results suggest that SsTrr1 encodes a TrxR that is of great important for oxidative stress tolerance, virulence, and sclerotial development of S. sclerotiorum.

Keywords: Sclerotinia sclerotiorum, thioredoxin reductase, oxidative stress, sclerotia, virulence, gene silencing

INTRODUCTION

Sclerotinia sclerotiorum is an ascomycete plant pathogen with a worldwide distribution (Bolton et al., 2006). This fungus infects more than 400 known plants and is the causal agent of stem rot in oilseed rape. S. sclerotiorum produces sclerotia, which are hard, asexual, resting structures. As melanized hyphal aggregates, sclerotia can survive for years in soil and play an important role in the disease cycle (Bolton et al., 2006; Erental et al., 2008). Sclerotia may germinate carboxigenically to produce millions of airborne ascosporas, which are the primary sources of inocula in most Sclerotinia diseases. Under certain conditions, sclerotia also germinate myceliogenically to produce hyphae, which can directly infect the hosts’ stem or leaves (Schwartz and Steadman, 1978; Bardin and Huang, 2001).

Reactive oxygen species (ROS), including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and the hydroxyl radical (-OH) play important roles as secondary messengers in many intracellular signaling pathways (Mittler et al., 2011; Ray et al., 2012). However, high ROS concentrations can
lead to DNA damage, protein inactivation and fragmentation, and lipid peroxidation (Aguirre et al., 2006). In plant–microbe interactions, ROS works as part of a defense mechanism and is a characteristic feature of the hypersensitive response (HR) (Lamb and Dixon, 1997). To detoxify ROS efficiently, cells usually use complex antioxidant responses, which mainly include superoxide dismutases, catalases, peroxidases, glutathione peroxidases, peroxiredoxins, and thioredoxins (Trxs) (Pomposiello et al., 2001; Aguirre et al., 2006).

The Trx system is ubiquitous from eukaryotes to archaea and plays a basic role in the maintenance of the redox environment in cells (Arné and Holmgren, 2000). The Trx system is composed of Trx, thioredoxin reductase (TrxR), and nicotinamide adenine dinucleotide phosphate (NADPH) (Holmgren, 1989). Trx contains a dithiol/disulfide active site (CGPC) and works as a major cellular disulfide reductase. Using NADPH as an electron donor, TrxR catalyzes the reduction of the active disulfide site in oxidized TrxR, Trx-S2, to a dithiol in reduced TrxR, Trx-(SH)2 (Arné and Holmgren, 2000). Reduced Trx directly reduces the disulfide in target proteins, and this process is required for several intracellular processes (Thöen et al., 2007). In addition to Trx, TrxR also has other substrates, such as the glutaredoxin-like protein, NrdH, in Escherichia coli (Jordan et al., 1997).

TrxR are homodimeric flavoenzymes that belong to a larger family of pyridine nucleotide-disulfide oxidoreductases. They contain an active redox disulfide and binding sites for flavin adenine dinucleotide (FAD) and NADPH in each subunit (Thöen et al., 2007). TrxR can be divided into two classes according to the molecular weight (Ghisla and Massey, 1989). High molecular weight TrxR is present in higher eukaryotes and has a molecular weight of 55–58 kDa, while low molecular weight TrxR (homodimers of 35–36 kDa subunits) is present in prokaryotes, archaea, plants, and fungi (Thöen et al., 2007).

Recently, some fungal genes that encode TrxR have been cloned and functionally analyzed. Two TrxRs (Trr1 and 2) that contain two TrxR genes that play distinct roles in the redox system and host infection (Zhang et al., 2016). Some evidence has shown that the TrxRs are required for pathogenic activity of S. sclerotiorum and pathogenicity of S. sclerotiorum were characterized. The findings could help to advance our understanding of the role of TrxR in fungal plant pathogens and the molecular mechanisms that are involved in the sclerotial development and pathogenicity of S. sclerotiorum.

MATERIALS AND METHODS

Fungal Strains and Culture Conditions

The S. sclerotiorum isolate “1980” (Godoy et al., 1990) was used as the wild-type strain in this study. Strains were routinely cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit) at 20°C. Transformants were cultured on PDA supplemented with hygromycin B at 100 µg/mL (Calbiochem, Riverside, CA, United States). The effects of auranofin (MedChem Express, Princeton, NJ, United States) on hyphae growth and sclerotial development were by adding a range of concentrations of auranofin (0–62.5 µM) to the PDA medium.

Vector Construction and Transform

An SsTrr1 gene-silencing vector was constructed based on plasmid pCit (Yu et al., 2012). The primer pairs STTrr1IaI (CGCATCGATTCAGCTCGCAGACTCGGTCT)/SiTrr1EcoRV (CGCGATATCTCGTTCCGGGCTTGGTTAC) and STTrr1 BamHI (CGCGGATCCCTCGACTCGCAGACTCGGTCT)/STTrr1 PstI (CGCGTGCAGTCTCGTTCCGGGCTTGGTTAC) were designed according to SsTrr1 cDNA sequences and then used to amplify the sense and antisense fragments of SsTrr1, respectively. The sense and antisense fragments were successfully inserted into the corresponding multiple cloning sites of the pCit vector. A hygromycin resistance gene was then inserted into the XbaI site of the newly constructed vector to create the SsTrr1 RNA silencing vector pSsTrr1. The vector was then linearized with XhoI and used to transform the wild-type protoplasts of S. sclerotiorum according to the method used by Rollins (2003).

Nucleic Acid Manipulation and Real-Time RT-PCR

To assay the expression levels of SsTrr1 transcripts in different stages of sclerotial development of S. sclerotiorum, the wild-type strain was cultured on cellophane over PDA, and mycelia were harvested at 2 days post-inoculation (dpi) (hyphae), 3 dpi (initial sclerotia), 5 dpi (developing sclerotia), and 8 dpi (mature sclerotia). The mature sclerotia were cultured on the surface of moist sand at 16°C and collected once the stipe initials appeared. To evaluate the expression levels of SsTrr1 transcripts in different transforms containing pSsTrr1, the wild-type strains and the transforms were cultured on PDA for 3 days. To analyze the expression levels of SsTrr1 under oxidative stress conditions, the wild-type strain was cultured on PDB for 1 day, and then the culture was treated with 10 mM H2O2 for 1 day. The RNA products in different samples were extracted with a Trizol reagent (TianGen, Dalian, China). First-strand cDNA synthesis was performed using a ReventAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Flamborough,
ON, Canada). The relative expression levels of SsTrr1 were obtained with real-time reverse-transcriptase polymerase chain reaction (RT-PCR) using a CFX96™ Real-time System (BioRad, Hercules, CA, United States). Real-time RT-PCR assays were performed according to Yu et al. (2012) with primer pair RT-SsTrr1Fp (AGAATTCCCTGTTTTCCCTAA)/RT-SsTrr1rp (GTGTTCGTCCTTGTCATCCATT), which was designed based on the cDNA of SsTrr1. The β-tubulin gene tub1 (SS1G_04652) was used as an internal control and amplified with the primer pair RT-tubfp (GTGAGGCTGAGGGCTGTGA)/RT-tubrp (CCTTTGGCGATGGAGG).

Pathogenicity Assay
Pathogenicity assays were conducted on Arabidopsis thaliana col-0 and Nicotiana benthamiana according to Yu et al. (2017) with slight modification. All plants were grown in a greenhouse at 25°C under a 16-h light/8-h dark cycle. The plants or leaves were inoculated with mycelium agar plugs (6 mm in diameter) obtained from the edges of the growth colony of the wild-type strain and SsTrr1 gene-silenced strains. Photographs were taken at 24 and 48 hpi for N. benthamiana and A. thaliana, respectively. The experiment was repeated at least three times, and each strain was evaluated with at least five plants with three leaves (15 leaves total).

Oxidative Stress Treatment
In order to test the effects of oxidative stress on hyphal growth, the wild-type strain and SsTrr1 gene-silenced strains were cultured on PDA medium and PDA with H₂O₂ (5, 10 mM). The colony diameters were then measured at 36 h to determine the inhibition of hyphal growth. Each experiment was repeated at least three times.

Thioredoxin Reductase Activity Measurements
The wild-type strain and SsTrr1 gene-silenced strains were cultured on PDA medium for 3 days. The total proteins for each strain were then extracted and used for the measurement of TrxR activities using a 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay with assay kits (Solarbio, Beijing, China). Absorbance values were monitored at 412 nm. TrxR activity levels were expressed as U/g mycelium. The unit U/g refers to the amount of TrxR in a 1-g sample that catalytic reduction of 1 μmol DTNB per minute.

RESULTS
Auranofin Inhibits the Hyphal Growth and Sclerotial Development of S. sclerotiorum
In order to determine whether TrxR activity is involved in the development of S. sclerotiorum, the effects of the TrxR inhibitor auranofin on the hyphal growth and sclerotal formation were examined. The results showed that hyphal elongation was inhibited in the presence of auranofin in PDA medium (50% effective concentration [EC₅₀] = 4.4 μM) (Figure 1A). The number of sclerotia was negatively correlated with increasing concentrations of auranofin (Figure 1B), suggesting that auranofin inhibited S. sclerotiorum sclerotal formation. These results reveal the inhibitory effect of auranofin on S. sclerotiorum and support the possibility that TrxR is required for the hyphal growth and sclerotal development of this fungus.

Characterization of Thioredoxin Reductase Gene in S. sclerotiorum
In order to explore the roles of TrxR in the development and pathogenicity of S. sclerotiorum, one candidate TrxR-encoded gene (SS1G_05899) was identified using the genome sequence of S. sclerotiorum (Amselem et al., 2011). The gene comprises five exons encoding a 346-amino-acid polypeptide, which contain a TRX reductase domain at amino acid positions K₄ to L₃₀₆ (E-value = 2.18e−145) according to a Conserved Domain Database (CDD) analysis (Marchler-Bauer et al., 2017). The sequence alignment demonstrated that the protein exhibited greater similarity with B. cinerea BcTrr1 (97% identity and 100%
query coverage) (Viefhues et al., 2014), *M. grisea* MgTRR1 (82% identity and 96% query coverage) (Fernandez and Wilson, 2014), *S. cerevisiae* ScTRR1 (67% identity and 94% query coverage), and ScTRR2 (66% identity and 99% query coverage) (Pearson and Merrill, 1998; Pedrajas et al., 1999) (Figure 2). Thus, the gene was named *SsTrr1* (*S. sclerotiorum* TrxR 1). To provide initial insight into the role of TrxR in fungal development, a real-time RT-PCR analysis was used to measure the abundance of *SsTrr1* mRNA in different growth stages of *S. sclerotiorum*. As shown in Figure 3, *SsTrr1* exhibited constitutive expression at different sclerotia development stages. However, the expression levels in the hyphae were twice that seen during the sclerotial development.

**Functional Analysis of *SsTrr1* in *S. sclerotiorum***

The gene-silenced vector pSiTrr1 was constructed as described in the section “Materials and Methods” in order to functionally analyze *SsTrr1* in *S. sclerotiorum*. The vector was used to transform the wild-type strain 1980 as described by Rollins (2003), and several transforms were obtained. *SsTrr1* expression levels in several different randomly selected transformants were determined with real-time RT-PCR, and *SiTrr1*-54 and *SiTrr1*-59 showed a dramatic decrease in the abundances of *SsTrr1* mRNA (Figure 4). Thus, these two strains were chosen for further study. The wild-type strain and *SsTrr1* gene-silenced strains were cultured for 3 days, and the TrxR activity in the mycelium was then determined using a DTNB assay. The results showed that the activities of TrxR in *SiTrr1*-54 and *SiTrr1*-59 were significantly decreased in comparison with that in the wild-type strain (Figure 5). This suggests that the inhibition of the *SsTrr1* expression levels leads to reduced TrxR activities in *S. sclerotiorum*.

**SsTrr1** in Relation to Sclerotia Development

When cultured on PDA plates, the two *SsTrr1* gene-silenced strains showed similar morphology of the hyphal branch to that of the wild-type strain (data not shown). However, the *SsTrr1* gene-silenced strains produced less of the slightly bigger sclerotia that formed in a random manner in the

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**Figure 2** Clustal X alignment of the amino acid sequence of *SsTrr1* with several reported TrxRs of different fungi, including *M. oryzae* TRR1 (EHA54395.1), *B. cinerea* BcTrr1 (XP_001560033.1), *S. cerevisiae* Trr1 (KZV12592.1), and Trr2 (KZV12592.1). Shading indicates sequence similarities of 100 (dark), 75 (medium), and 50% (light). Numbers mean the amino acid of the predicted polypeptide.
SsTrr1 Related to Pathogenicity

In order to determine the effects of SsTrr1 silencing on the pathogenicity of S. sclerotiorum, detached A. thaliana leaves were inoculated with mycelium plugs of SiTrr1 gene-silenced strains. As demonstrated in Figure 7A, SiTrr1-54 and SiTrr1-59 led to small lesions on the A. thaliana leaves compared to the wild-type strain. The pathogenicity of the two gene-silenced strains was also tested on intact N. benthamiana plants, and smaller lesions were observed on the leaves (Figure 7B). These results indicate that SsTrr1 is required for the full virulence of S. sclerotiorum.

SsTrr1 in Relation to Oxidative Stress Tolerance

The relative SsTrr1 expression levels under oxidative stress were determined via real-time RT-PCR in order to extensively characterize the role of SsTrr1 in response to oxidative stress in S. sclerotiorum. As shown in Figure 8A, the SsTrr1 expression level increased sharply in hyphae that were treated with 10 mM H2O2. The hyphal growth under oxidative stress between the wild-type and SsTrr1 gene-silenced strains was then compared. When growth on PDA plates was amended with H2O2, hyphal growth inhibition was significantly greater for SiTrr1-54 and SiTrr1-59 than the wild-type strain (Figure 8B). The results indicate that SsTrr1 contributes to the oxidative stress tolerance in S. sclerotiorum.

DISCUSSION

In this study, the gene SsTrr1, which encodes a TrxR in S. sclerotiorum, was cloned and functionally analyzed. SsTrr1 was shown to have an effect on the oxidative stress tolerance, sclerotial development, and pathogenicity of S. sclerotiorum.

Evidence has shown that the numbers of TrxRs vary among different fungi. In S. cerevisiae, two TrxRs (Trr1 and 2) were identified and were shown to be located in the cytoplasm and mitochondria, respectively (Pearson and Merrill, 1998; Pedrajas et al., 1999). The filamentous fungal insect pathogen B. bassiana also contains two TrxRs that play distinct roles in the redox system and host infection (Zhang et al., 2016). However, only one TrxR was identified in fungal plant pathogens, including B. cinerea, M. oryzae, and A. alternata, and a cytoplasmic location

plates (Figures 6A,B). The average numbers and dry weights of the sclerotia produced by SiTrr1-59 per 9-cm plate were approximately 74 and 110% of those produced by the wild-type strain, respectively (Figures 6C,D). The results were consistent with previous findings of sclerotial formation being inhibited by auranofin, suggesting that SsTrr1 is related to sclerotial development in S. sclerotiorum.
was demonstrated in these fungi (Fernandez and Wilson, 2014; Viefhues et al., 2014; Ma et al., 2018). BLASTP searches indicated that S. sclerotiorum contains only one TrxR-encoding gene, and this number is consistency with other fungal plant pathogens. Since there is a lack of effective fluorescent protein labeling methods, the subcellular localization of TrxR in S. sclerotiorum was predicted using ProtCom 9.0 servers¹, and the results showed that SsTrr1 is most likely localized in the cytoplasm.

Some genes that are related to ROS modulation in S. sclerotiorum have been discussed. Functional loss of a Cu/Zn superoxide dismutase in S. sclerotiorum resulted in an increase in sensitivity to oxidative stress in culture (Veluchamy et al., 2012; Xu and Chen, 2013). However, deletion of the catalase, SCAT1, in S. sclerotiorum led to an increase in tolerance to H$_2$O$_2$, indicating that SCAT1 is not essential for H$_2$O$_2$ degradation in vitro (Yarden et al., 2014). In recent years, several fungal TrxR-encoding genes have been shown to play critical roles in oxidative stress responses (Fernandez and Wilson, 2014; Viefhues et al., 2014; Ma et al., 2018). In this study, SsTrr1 showed a sharp increase in expression under oxidative stress conditions, and the gene-silenced strains exhibited sensitivity to H$_2$O$_2$, suggesting a conserved function for TrxR in oxidative stress in fungi.

The generation of ROS has been recorded as one of the earliest resistance responses for plants against fungal pathogens (Bolwell et al., 1995). ROS detoxification and tolerance are critical for S. sclerotiorum hyphae to infect host plants successfully (Kim et al., 2011; Williams et al., 2011; Yarden et al., 2014). Previous evidence has shown that these genes, which play critical roles in the detoxification and tolerance of ROS, are essential for S. sclerotiorum pathogenesis (Veluchamy et al., 2012; Xu and Chen, 2013; Yu et al., 2015). McLoughlin et al. (2018) reported that foliar applications of dsRNA-targeted SsTrr1 reduced S. sclerotiorum infection in B. napus. In this study, we found that SsTrr1 gene-silenced strains exhibited attenuated virulence in different hosts. SiTrr1-59 with a lower expression level of SsTrr1 led to smaller lesions. These data further indicated that TrxR is critical for the successful infection of this fungus. The role of TrxR in S. sclerotiorum virulence is consistent with those of fungal plant pathogens such as B. cinerea (Viefhues et al., 2014), A. alternata (Ma et al., 2018), and M. oryzae (Fernandez and Wilson, 2014), in addition to the fungal insect pathogen

¹http://www.softberry.com/
FIGURE 7 | Pathogenicity analysis of SsTrr1 gene-silenced strains. Detached leaves of A. thaliana (A) and intact N. benthamiana plants (B) were inoculated with PDA plugs colonized with the wild-type strain, SiTrr1-54 and SiTrr1-59. Lesion size were measured at 24 and 48 hpi for N. benthamiana and A. thaliana, respectively. Error bars indicate standard deviation. Statistical significance is indicated in the graph (one-way ANOVA). *P < 0.05.

FIGURE 8 | Functional analysis of SsTrr1 in oxidative stress response. (A) SsTrr1 transcript in hyphae treated with 10 mM H$_2$O$_2$. Total SsSvf1 cDNA abundance in the samples was normalized to using tub1 gene as a control. The relative expression of SsTrr1 in the untreated strain was set as 1. Bars indicate standard error. (B) Percent growth inhibition of wild-type strain and SsTrr1 gene-silenced strains on PDA medium with 5 and 10 mM H$_2$O$_2$. Bars indicate standard deviation. Asterisks denote significant differences (one-way ANOVA). **P < 0.01.
S. sclerotiorum via intracellular redox-level regulation. Activity is critical for sclerotial development. Since TrxR is the level of TrxRs are required for fungal sclerotial development. However, fewer of sclerotia. To our knowledge, this is the first report that silencing of the TrxR-encoding gene auranofin, which inhibited TrxR activity. Furthermore, targeted silencing of the NADPH oxidase genes SsNox1 and SsNox2 resulted in reduced ROS levels and limited sclerotial development of S. sclerotiorum (Kim et al., 2011). However, a Cu/Zn superoxide dismutase gene-deletion mutant exhibited normal sclerotial formation (Xu and Chen, 2013), indicating a complex role for ROS in the sclerotial development of S. sclerotiorum.

In this study, sclerotial development was suppressed by auranofin, which inhibited TrxR activity. Furthermore, targeted silencing of the TrxR-encoding gene SsTrr1 led to the production fewer of sclerotia. To our knowledge, this is the first report that TrxRs are required for fungal sclerotial development. However, the level of SsTrr1 expression exhibited a decrease when sclerotia began to form, which indicated a dynamic balance of TrxR activity is critical for sclerotial development. Since TrxR is critical and consists of Trx systems that act against oxidative stress promoted sclerotial metamorphosis (Georgiou et al., 2006; Papapostolou et al., 2014). Silencing of the NADPH oxidase genes SsNox1 and SsNox2 resulted in reduced ROS levels and limited sclerotial development of S. sclerotiorum (Kim et al., 2011). However, a Cu/Zn superoxide dismutase gene-deletion mutant exhibited normal sclerotial formation (Xu and Chen, 2013), indicating a complex role for ROS in the sclerotial development of S. sclerotiorum.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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