Received: 2 February 2021 | Revised: 22 July 2021 | Accepted: 4 August 2021

DOI: 10.1111/mpp.13127

ORIGINAL ARTICLE

Molecular Plant Pathology WILEY

Sclerotinia sclerotiorum Thioredoxin1 (SsTrx1) is required for pathogenicity and oxidative stress tolerance

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Funding information
Natural Science Foundation of Chongqing, Grant/Award Number: cstc2019jcyj-zdxmX0012 and cstc2019jcyj-msxmX0486; Fundamental Research Funds for the Central Universities, Grant/Award Number: XDJK2018AA004, XDJK2018B022 and SWU120075; National Science Foundation of China, Grant/Award Number: NSFC 31801395 and 31971978

Abstract

Sclerotinia sclerotiorum infects host plant tissues by inducing necrosis to source nutrients needed for its establishment. Tissue necrosis results from an enhanced generation of reactive oxygen species (ROS) at the site of infection and apoptosis. Pathogens have evolved ROS scavenging mechanisms to withstand host-induced oxidative damage. However, the genes associated with ROS scavenging pathways are yet to be fully investigated in *S. sclerotiorum*. We selected the *S. sclerotiorum Thioredoxin1* gene (*SsTrx1*) for our investigations as its expression is significantly induced during *S. sclerotiorum* infection. RNA interference-induced silencing of *SsTrx1* in *S. sclerotiorum* affected the hyphal growth rate, mycelial morphology, and sclerotial development under in vitro conditions. These outcomes confirmed the involvement of *SsTrx1* in promoting pathogenicity and oxidative stress tolerance of *S. sclerotiorum*. We next constructed an *SsTrx1*-based host-induced gene silencing (HIGS) vector and mobilized it into *Arabidopsis thaliana* (HIGS-*A*) and *Nicotiana benthamiana* (HIGS-*N*). The disease resistance analysis revealed significantly reduced pathogenicity and disease progression in the transformed genotypes as compared to the nontransformed and empty vector controls. The relative gene expression of *SsTrx1* increased under oxidative stress. Taken together, our results show that normal expression of *SsTrx1* is crucial for pathogenicity and oxidative stress tolerance of *S. sclerotiorum*.

KEYWORDS
host-induced gene silencing, oxidative stress, reactive oxygen species, RNAi, *Sclerotinia sclerotiorum*, thioredoxin

1 | INTRODUCTION

Sclerotinia stem rot, caused by the necrotrophic fungus *Sclerotinia sclerotiorum*, is a destructive plant disease that causes yield losses in vegetable and oilseed brassicas worldwide (Bolton et al., 2006). It is difficult to control *S. sclerotiorum* as it produces asexual, hard, resting sclerotia that can survive for many years in the soil. These germinate under favourable conditions to initiate a new cycle of the disease (Erental et al., 2007; Xu et al., 2018). Current methods of pathogen control do not provide reliable protection from the disease. It is thus important to develop new management strategies that offer sustainable disease control. Minimizing damage by deploying cultivars with a genetically inherited reduction in the susceptibility to pathogens is the preferred form of disease management. Pathogens in turn

employ multiple strategies to undermine the defence responses of the host plants. *S. sclerotiorum* secretes hydrolytic cell wall-degrading enzymes, such as endo- (Sspg1, Sspg3, Sspg5, and Sspg6) and exo- (Ssxpg1 and Ssxpg2) polygalacturonases (Bashi et al., 2012; Li et al., 2004; Yu et al., 2017). The pathogen also secretes oxalic acid (OA), which promotes pathogenicity by interfering with the redox environment and pH signalling in the host. *S. sclerotiorum* mutants that fail to synthesize oxalates are nonpathogenic (Cessna et al., 2000). Revertants with restored oxalate biosynthesis regain their virulence (Godoy et al., 1990). OA promotes disease development by eliciting programmed cell death (Kim et al., 2008; Liang et al., 2015; Williams et al., 2011; Yu et al., 2017). Targeted mutagenesis and gene silencing experiments have confirmed the roles of genes encoding oxaloacetate acetylhydrolase (*Ssoah1*) (Li et al., 2018; Liang et al., 2015; Xu et al., 2015), arabino furanosidase/β-xylosidase precursor (*Ssaxp*), and an endo-β-1,4-xylanase (*SsXyl1*) in *S. sclerotiorum* virulence (Yajima et al., 2009; Yang et al., 2018). Functional analysis of *S. sclerotiorum* secretomes has also shown the involvement of cell hydrolysis, oxidation–reduction processes, and the redox state in the processes associated with host infection (Heard et al., 2015).

Reactive oxygen species (ROS) such as superoxide (O_2^-), singlet oxygen (O_2), hydroxyl radical (·OH), hydroperoxyl radical (HO_2), and hydrogen peroxide (H_2O_2) are among the primary defence responses that are triggered by the plants in response to pathogen attack (Bolwell et al., 1995; Ding et al., 2020; Gill & Tuteja, 2010; Jones & Dangl, 2006; Mittler, 2017). A basal level of ROS is essential for cellular proliferation and other physiological functions in plants. In contrast, elevated ROS levels cause cellular injury, DNA damage, protein inactivation, fragmentation of macromolecules, and apoptosis (Aguirre et al., 2005; Foyer & Noctor, 2013; Mittler, 2017). ROS production is triggered by increased enzymatic activities of plasma membrane-bound NADPH oxidases, catalases, glutathione peroxidases, and thioredoxins (Trxs) (Aguirre et al., 2005; Lamb & Dixon, 1997). Both hosts and pathogens have evolved ROS scavenging systems to reduce cellular damage and sustain ROS balance (Ding et al., 2020). For instance, enzymes such as catalases, peroxidases, ascorbate peroxidase (APX), glutathione *S*-transferase (GST), superoxide dismutase (SOD), and tripeptide glutathione (GSH) are key ROS scavengers in plants (Ding et al., 2020; Pomposiello et al., 2001; Yan et al., 2008).

Trx, Trx reductase (TrrR), and nicotinamide adenine dinucleotide phosphate (NADPH) are key elements of the Trx system, which is associated with redox regulation and antioxidant defence in all living beings (Arnér & Holmgren, 2000; Zhang et al., 2019). These act at both intracellular and extracellular levels and are localized in the cytosol, mitochondrion, plastid, and nucleus (Hofmann, 2010; Liebthal et al., 2018). They are substrates for reductive enzymes such as peroxidases and ribonucleotide reductase (Arnér & Holmgren, 2000; Gelhaye et al., 2004; Montrichard et al., 2009). Trx is crucial for growth, development, stress sensing, repair, cellular redox homeostasis, enzymatic activation, and protection from oxidative stresses (Arnér & Holmgren, 2000; Holmgren, 1989; Liebthal et al., 2018; Meyer et al., 2005, 2008, 2009, 2012). The Trx system acts by reducing oxidized cysteine groups of proteins to form a disulphide bond that is reduced by TrrR and NADPH (Arnér & Holmgren, 2000; Zhang et al., 2019). Trxs are classified as m, f, x, y, o, and h based on their function and localization within the plant cells (Gelhaye et al., 2004). Nuruzzaman et al. (2012) have identified various Trx genes based on genome-wide expression analysis under various biotic and abiotic stresses. Pathogens employ the Trx system to defend against oxidative stress imposed by the host. Many fungal *TrrR* genes have been functionally characterized (Fernandez & Wilson, 2014; Pedrajas et al., 1999). *TrrR* is crucial for sclerotial development and cellular redox regulation during *S. sclerotiorum* infection (Zhang et al., 2019). Despite these studies, there is no published evidence for assigning functions and biological roles to the *Trx* gene, specifically in pathogenicity, virulence, and oxidative stress tolerance, in *S. sclerotiorum*. RNA interference (RNAi) and host-induced gene silencing (HIGS) are also widely used for functional genomics (Baulcombe, 2015; Hu et al., 2015; Seifbarghi et al., 2017; Wang et al., 2019; Yu et al., 2017; Zhang et al., 2018). Movement of RNA between plant hosts and invading pathogens is an important step for RNAi-mediated HIGS and cross-kingdom RNAi (Hua et al., 2018; Wang & Dean, 2020; Weiberg et al., 2015).

Here, we report the functional validation of *SsTrx1* for its role in *S. sclerotiorum* pathogenicity and virulence through RNAi-mediated gene silencing and HIGS. Silencing of *SsTrx1* adversely affected the progression of disease and sclerotial development in *Arabidopsis thaliana* and *Nicotiana benthamiana*. Our study improves our understanding of the *S. sclerotiorum* pathosystem and also opens up the possibility of using HIGS as a method of choice for *S. sclerotiorum* management in field crops.

2 | RESULTS

2.1 | Characterization of the *SsTrx1* gene and sequence analysis

SsTrx1 (*SS1G-08534*), which encodes Trx1, has a nucleotide length of 794 bp, with two intronic regions. It has a mature transcript length of 441 bp and encodes a 146-amino-acid protein. Homologous *Trx* gene sequences from various fungal and plant species were retrieved using the BLAST function from NCBI (https://www.ncbi.nlm.nih.gov/gene/?term). Multiple sequence alignment and phylogenetic tree construction revealed 83.83% similarity (48% query coverage) with the previously reported *Botrytis cinerea Trx1* gene (XM_001546556.2) sequence (Amselem et al., 2011; Kan et al., 2017). However, *SsTrx1* showed low sequence identity and query coverage with the corresponding genes from other plant species and/or fungal species (Figure S2a,b). Similarity percentages and query coverages ranged from 74.03% similarity (17% query coverage) with *A. thaliana* to 82.67% (17%) with *Nicotiana* sp., 74.68% (17%) with *Brassica napus*, 73.19% (52%) with *Aspergillus campestris*, 73.55% (69%) with *Glarea lozoyensis*, and 74.89% (50%) with *Sphaerulina musiva*. *SsTrx1* shared no similarity with the previously reported *S. sclerotiorum TrrR1* gene (Zhang et al., 2019).

2.2 | The *SsTrx1* gene is highly expressed at initial developmental stages of *S. sclerotiorum*

Quantitative reverse transcription PCR (RT-qPCR) of the *SsTrx1* gene in advanced developmental stages of *S. sclerotiorum* growth on potato dextrose agar (PDA) revealed induction of *SsTrx1* transcription at the hyphal stage (2 days postinoculation [dpi]). Gene expression was high at the sclerotial initiation stage (3 dpi). This was followed by a gradual decline in gene expression from 5 dpi (developing sclerotia). The lowest *SsTrx1* expression levels were recorded at 7 dpi (mature sclerotia stage; Figure 1). Apparently, *SsTrx1* is highly expressed in the initial development stages of growth.

2.3 | Involvement of *SsTrx1* in pathogenicity of *S. sclerotiorum*

Several RNAi *S. sclerotiorum* transformants were raised on PDA fortified with hygromycin. Compared to the *S. sclerotiorum* wild-type (WT) strain 1980, the relative expression of the *SsTrx1* gene was significantly lower in the *SsTrx1* gene-silenced transformants designated as *SsTrx1-01*, *SsTrx1-02*, and *SsTrx1-03* in the T_2 generation (Figure 2). Only these transformants were retained for further experiments. For pathogenicity assays, we inoculated leaves and/or stems of *A. thaliana*, *B. napus*, and *N. benthamiana* with WT and strains *SsTrx1-01*, *SsTrx1-02*, and *SsTrx1-03*. Significant differences were recorded for the size of lesions produced on *A. thaliana* leaves ($n = 27$; $df = 8, 3$; $p < .01$), *B. napus* leaves ($n = 60$; $df = 19, 3$; $p < .01$), *B. napus* stems ($n = 30$; $df = 9, 3$; $p < .01$), and *N. benthamiana* leaves ($n = 30$; $df = 9, 3$; $p < .01$) inoculated with WT and *SsTrx1* gene-silenced strains (Tukey post hoc test), where *n* represents the total number of observations and *df* represents the degrees of freedom (Figure 3a–h). At 24 hr postinoculation (hpi), the WT produced lesions with an average size of around 0.577 cm^2, while strains *SsTrx1-01* to *SsTrx1-03* produced lesions ranging in size from 0.140 to 0.180 cm^2 on *A. thaliana* detached leaves. At 48 hpi, WT produced lesions of approximately 0.249 and 8.569 cm^2 and strains *SsTrx1-01* to *SsTrx1-03* produced lesions ranging in size from 0.067 to 0.109 cm^2 and from 3.316 to 4.372 cm^2 on *B. napus* and *N. benthamiana* detached leaves, respectively. On *B. napus* detached stems, WT produced lesions with a mean length of approximately 3.44 cm, while strains *SsTrx1-01* to *SsTrx1-03* produced lesions ranging in size from 1.38 to 1.61 cm at 48 hpi (Figure 3a–h). These results clearly confirmed the role of *SsTrx1* in the pathogenicity of *S. sclerotiorum*.

2.4 | Silencing of *SsTrx1* is related to sclerotial development in *S. sclerotiorum*

The WT and strains *SsTrx1-01* to *SsTrx1-03* (T_2 generation) were also inoculated into PDA to facilitate analysis of mycelial growth and morphology. The growth rates were significantly lower in *SsTrx1* gene-silenced strains than in the WT, and mycelial morphology of *SsTrx1-01*, *SsTrx1-02*, and *SsTrx1-03* was visibly different from that of the WT (Figure 4a,b). Moreover, significant differences were observed in the average number of sclerotia and sclerotial mass per plate ($n = 3$; $df = 2, 3$; $p < .05$) between

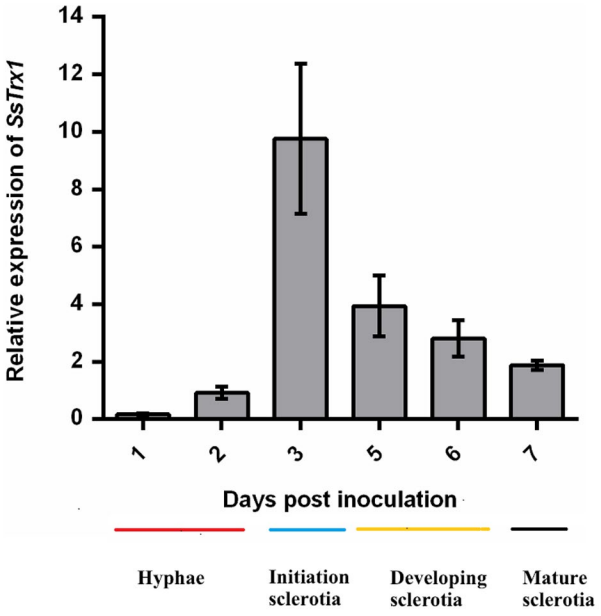

FIGURE 1 The relative gene expression level of *SsTrx1* in different sclerotial developmental stages as quantified by quantitative reverse transcription PCR. The *SsTrx1* cDNA was normalized to the *SsTubulin* cDNA. Bars indicate the standard error

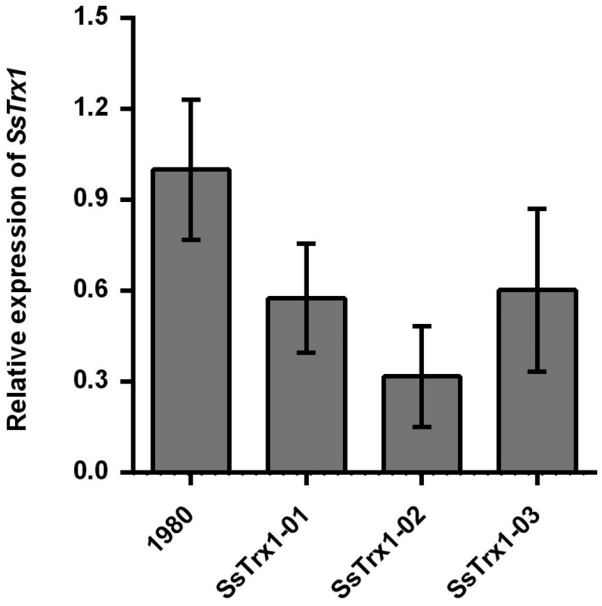

FIGURE 2 The gene expression of *SsTrx1* in RNAi transformants as determined by quantitative reverse transcription PCR. The *SsTrx1* cDNA was normalized to the *SsTubulin* cDNA. The amount of cDNA in *Sclerotinia sclerotiorum* strain 1980 was set as 1. Bars indicate the standard error

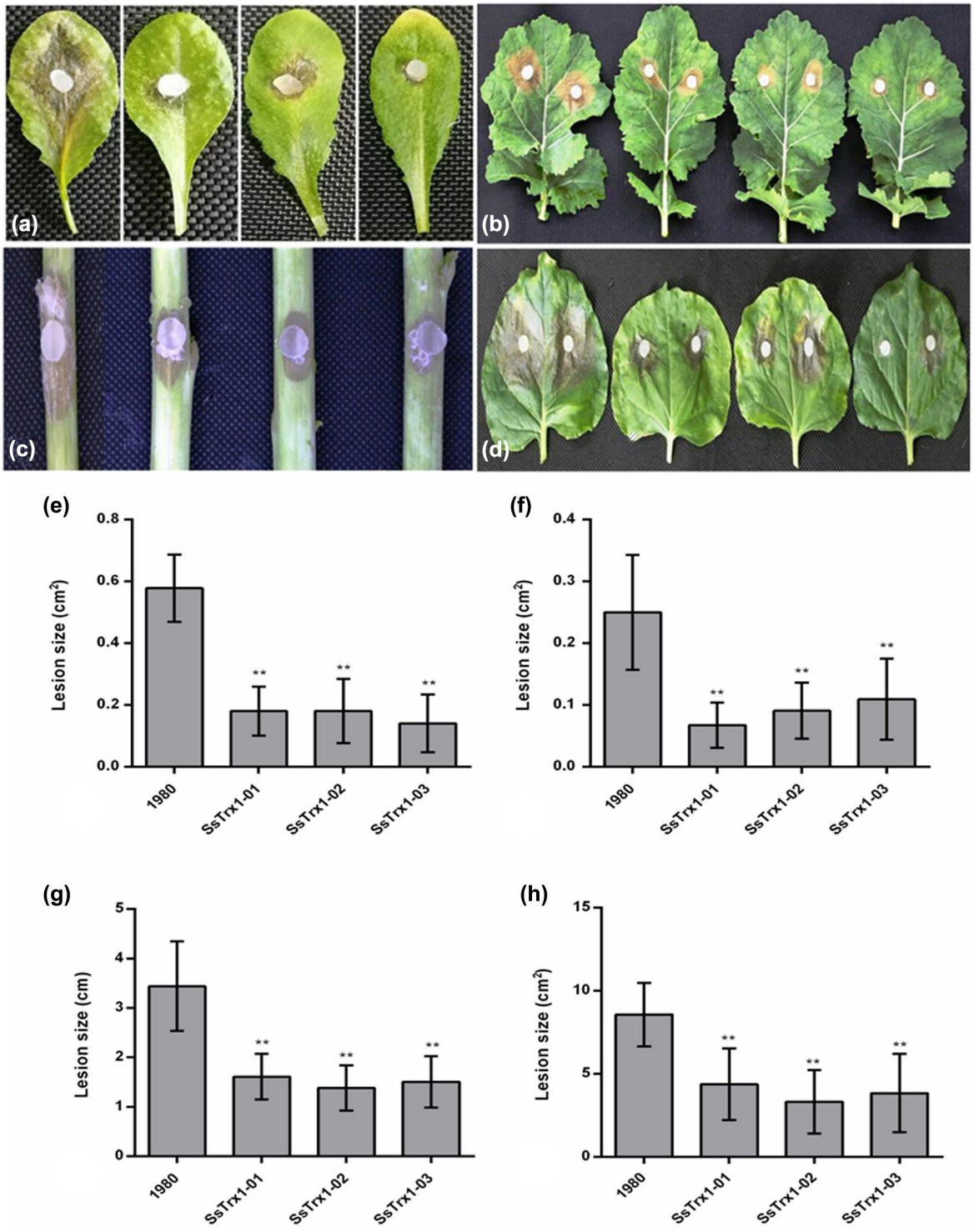

FIGURE 3 Pathogenicity analysis depicting detached (a) *Arabidopsis thaliana* (Col-0) leaves, (b,c) *Brassica napus* 'Zhongshuang 11' leaves and stems, and (d) *Nicotiana benthamiana* leaves, inoculated with *Sclerotinia sclerotiorum* strain 1980 (WT) and *SsTrx1* RNAi transformants *SsTrx1-01*, *SsTrx1-02*, and *SsTrx1-03*. (e–h) Mean lesion area (cm^2) in (e) *A. thaliana* leaves ($n = 27$; $df = 8, 3$; $p < .01$), (f) *B. napus* leaves ($n = 60$; $df = 19, 3$; $p < .01$), (g) *B. napus* stems ($n = 30$; $df = 9, 3$; $p < .01$), and (h) *N. benthamiana* leaves ($n = 30$; $df = 9, 3$; $p < .01$). The bars indicate standard deviation and asterisks (**) denote significant differences (one-way analysis of variance, Tukey post hoc test)

SsTrx1 gene-silenced strains and WT at 25 dpi. The average number of sclerotia was around 25 for *SsTrx1* gene-silenced strains, corresponding to a sclerotial mass of 0.306 ± 0.015 g (*SsTrx1-01*), 0.293 ± 0.005 g (*SsTrx1-02*), and 0.300 ± 0.010 g (*SsTrx1-03*). For WT, the number of sclerotia was around 36 per plate with a sclerotial mass of 0.253 ± 0.015 g (Figure 4c,d). Apparently, *SsTrx1* is associated with sclerotial growth and development in *S. sclerotiorum*.

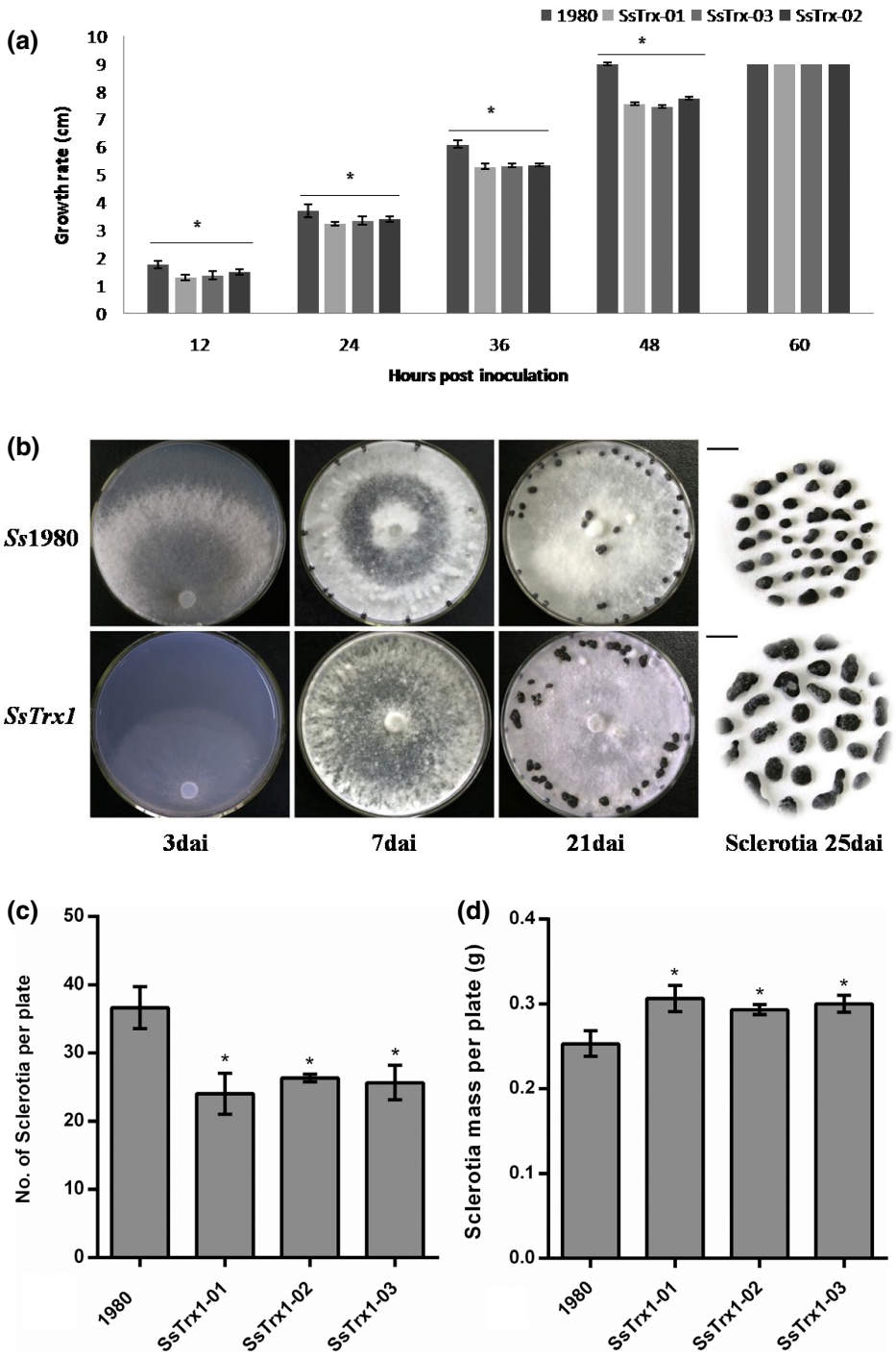

FIGURE 4 Mycelial growth and morphology of *Sclerotinia sclerotiorum* 1980 and *SsTrx1* RNAi transformants during the infection process in potato dextrose agar. (a) Growth rate. (b) Mycelial morphology. Scale bar = 1 cm. (c) Average number of sclerotia per plate. (d) Sclerotial weight per plate (g) in wild-type (WT) strain 1980 and *SsTrx1* RNAi transformants ($n = 3$; $df = 2, 3$; $p < .05$). The bars indicate standard deviation and asterisks * denote significant differences (one-way analysis of variance, Tukey post hoc test)

2.5 | HIGS in *N. benthamiana* and *A. thaliana* compromised pathogenicity of *S. sclerotiorum*

The HIGS-pBinGlyRed3-*SsTrx1* vector was transformed into ecotype Columbia (Col-0) (WT) *A. thaliana* plants. All transformants were analysed for the presence of the *SsTrx1* gene via PCR to rule out the possibility of gene silencing of the endogenous *Trx* gene in *A. thaliana*. Twenty-seven transgenic plants were confirmed positive. Significant differences in lesion sizes were also observed among 27 confirmed transgenic plants and WT in the T_1 generation (Figure S3). The lesion size in 27 transgenic plants varied from 0.016 to 0.557 cm^2, whereas the lesion size of WT was 0.745 ± 0.055 cm^2 at 24 hpi. In the T_2 generation, eight lines with single-copy inheritance were screened for virulence under both in vivo (intact leaf)

and in vitro (detached leaf) conditions at 24 hpi. Out of these, three transgenic lines (HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48*) were selfed to produce the T_3 generation for further virulence assays and gene expression analysis. In both in vivo (intact leaf) and in vitro (detached leaf) virulence assays, significant differences in lesion area were detected between (a) HIGS transgenic lines and (b) WT and empty vector (EV) controls ($n = 10$; $df = 4, 4$; $p < .01$; Tukey post hoc test) (Figure 5a,b). In the case of in vivo virulence assays, the mean lesion areas of WT and EV were 1.10 and 1.04 cm^2, respectively (Figure 5a [A–E]). In contrast, the lesion areas of HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48* varied from 0.26 to 0.49 cm^2 (Figure 5b).

For in vitro virulence assays, the mean lesion areas of WT and EV were 1.20 and 1.18 cm^2, respectively (Figure 5a: F–J). In contrast, the mean lesion areas of HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48* varied from 0.33 to 0.48 cm^2 (Figure 5b). These results reconfirmed that *SsTrx1* is required for pathogenicity of *S. sclerotiorum*. The gene expression level of *SsTrx1* in the WT inoculated into HIGS transgenic lines was reduced by 93.42%–96.16% compared with those of WT and EV plants at 24 hpi (Figure 5c). Similar findings were recorded for HIGS-*N* lines carrying the HIGS-pC2301M1B-*SsTrx1* vector. Significant differences were observed in lesion size of WT, EV, and HIGS-*N* after the Tukey post hoc test ($n = 20$; $df = 9, 2$; $p < .01$). The pathogenicity assays (detached leaf) showed a smaller lesion size (1.574 ± 1.20 cm^2) on the leaves of HIGS-*N* lines compared with WT and EV plants (6.274 and 5.424 cm^2, respectively) after 72 hpi (Figure 6a,b). There was a corresponding reduction (96.1%–96.3%) in the gene expression levels of *SsTrx1* in transformed leaves inoculated with *S. sclerotiorum* (Figure 6c). To rule out the effects of altered expression of *SsTrx1* on the activities of other host defence genes, we analysed the gene expression of *PR1*, *PR2*, *PR5*, and *PDF1.2* in HIGS-*A* transgenic plants in the T_3 generation at 0 hpi. No significant difference was observed in HIGS transgenic lines HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48* compared with WT and EV (Figure S4).

2.6 | *SsTrx1* is associated with oxidative stress tolerance in *S. sclerotiorum*

The Trx system is strongly activated under oxidative stress. To investigate oxidative stress tolerance, we compared hyphal growth and inhibition rates between WT and gene-silenced strains grown on PDA supplemented with 0, 5, 10, and 15 mM H_2O_2. The data were recorded at 12, 24, 36, 48, 60, and 72 hpi. Hyphal growth was slower in *SsTrx1* gene-silenced strains compared to the WT. Hyphal growth was completely subdued at 15 mM H_2O_2 in the *SsTrx1* transformant (Figure 7a). Significant differences were also observed in the inhibition rates at H_2O_2 concentrations of 5, 10, and 15 mM ($p < .01$). The inhibition rates were on the higher side for *SsTrx1* transformants compared with the WT (Figure 7b). The inhibition rates varied from 88.18% to 91.23% (15 mM H_2O_2) in *SsTrx1-01*, *SsTrx1-02*, and *SsTrx1-03*, compared to 64.12% in WT. The relative *SsTrx1* gene expression levels were higher in hyphae treated with 10 mM H_2O_2 compared with those treated with 5 mM H_2O_2 (Figure 7c).

The histochemical detection of H_2O_2 and O_2^- was performed with 3-3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining at 0, 6, and 12 hpi in *A. thaliana* WT and transgenic lines HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48*. The NBT hyphal staining indicated dark stained hyphae in *SsTrx1-01* gene-silenced transformants due to higher O_2^- accumulation compared to the lightly stained hyphae in *S. sclerotiorum* WT (Figure 7a). ROS detoxification was evident in *A. thaliana* HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48* transgenic lines as these failed to produce at 0 and 6 hpi. However, dark staining was observed in close proximity to the inoculation column of gene-silenced transgenics compared with relatively light staining observed in WT at 12 hpi (Figure S5). These results confirmed that the *SsTrx1* gene is related to O_2^- and H_2O_2 accumulation and plays a crucial role in oxidative stress tolerance in *S. sclerotiorum*.

3 | DISCUSSION

Necrotrophic pathogens infect plants by suppressing signalling pathways and optimizing nutrient acquisition through OA, ROS, and cell wall-degrading enzymes (Aguirre et al., 2005; Annis & Goodwin, 1997; Pomposiello et al., 2001). OA suppresses the oxidative burst in the host plant (Cessna et al., 2000), while ROS induces cellular damage and programmed cell death (Gill & Tuteja, 2010; Kariola et al., 2005; Lamb & Dixon, 1997; Sharma et al., 2012). Plants are damaged if ROS produced by the pathogen overwhelm the ROS scavenging capacity of plants (Sharma et al., 2012). *S. sclerotiorum* overcomes the host defences by detoxifying secondary metabolites (Stotz et al., 2011) and modulating oxidative stress with the help of Trx-interacting proteins (Ding et al., 2020; Xu & Chen, 2013; Yu et al., 2015; Zhang et al., 2019). The Trx system is critical for maintaining the cellular redox balance and antioxidant function and controlling cell death (apoptosis) following interaction with Trx-interacting proteins (Lu & Holmgren, 2012, 2013; Michelet et al., 2006). Trxs, peroxiredoxins, and glutaredoxins (also known as thioloxido-reductases) are activated during oxidative stress. These reduce oxidized proteins via cysteine thiol-disulphide exchange (Hanschmann et al., 2013; Liebthal et al., 2018; Meyer et al., 2012; Montrichard et al., 2009). Many fungal genes that encode TrrR have been cloned and functionally analysed in *Saccharomyces cerevisiae* (Pedrajas et al., 1999), *Alternaria alternata* (Ma et al., 2018), *B. cinerea* (Viefhues et al., 2014), and *S. sclerotiorum* (Zhang et al., 2019). Their suppression impaired the virulence and antioxidant capabilities of the host species. The *SsTrx1* gene is up-regulated at early stages of infection, but it attains maximum expression levels at the sclerotial initiation stage (Willetts & Bullock, 1992; Willets et al., 1980).

Most commercial cultivars of rapeseed-mustard are susceptible to *S. sclerotiorum* stem rot (Denton-Giles et al., 2018; Rana et al., 2017; Uloth et al., 2015). However, few sources of quantitative resistance have been reported (Atri et al., 2019; Boudhrioua et al.,

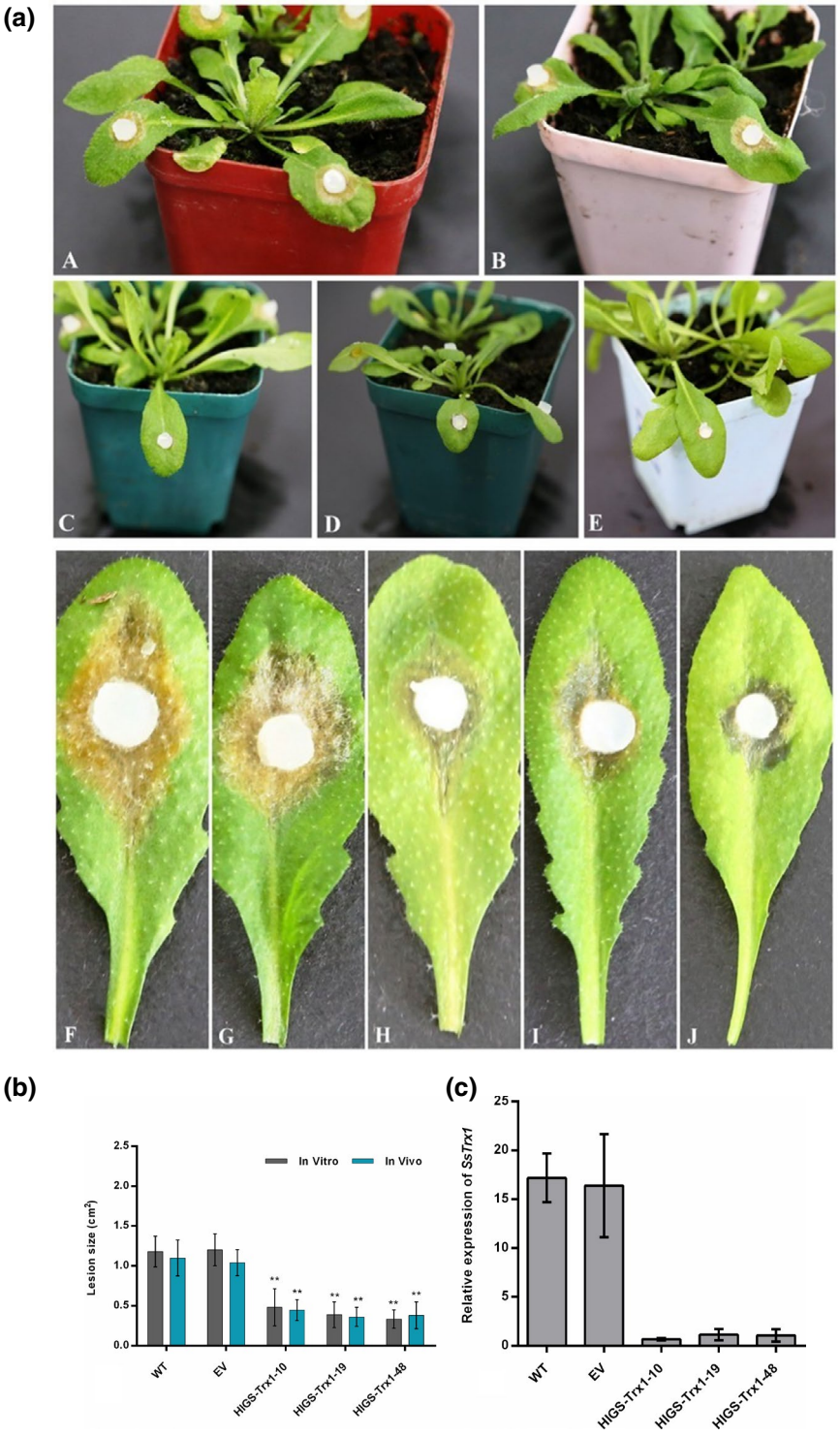

FIGURE 5 The disease resistance response of wild type (WT), empty vector (EV), and host-induced gene silencing (HIGS)-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48* against *Sclerotinia sclerotiorum* in the T_3 generation. (a) Pathogenicity assay (from left to right): (A) WT, (B) EV, and (C–E) HIGS-*Arabidopsis thaliana* transgenic lines under in vivo conditions and (F) WT, (G) EV, and (H–J) HIGS-*A. thaliana* transgenic lines under in vitro conditions at 24 hr postinoculation (hpi). (b) Lesion size (cm^2) of WT, EV, and HIGS-*A. thaliana* transgenic lines under in vivo and in vitro conditions ($n = 10$; $df = 4, 4$; $p < .01$) at 24 hpi. The bars indicate the standard error and asterisks ** denote significant differences (one-way analysis of variance) (Tukey post hoc test). (c) Relative gene expression of *SsTrx1* in gene-silenced transformants compared with WT and EV plants

2020; Rana et al., 2017, 2019; Sun et al., 2020; Taylor et al., 2015; Wei et al., 2014, 2016). The majority of these sources provide partial resistance and are isolate-specific (Garg et al., 2010; Sharma et al., 2018; Taylor et al., 2015). Diversity of pathogen isolates can be an issue for the wide applicability of a resistance source if it is isolate-specific. Population structure, haplotype diversity, and variation for aggressiveness have been widely reported for *S. sclerotiorum* isolates (Clarkson et al., 2017; Leyronas et al., 2018; Sharma et al., 2018; Yu et al., 2020). Canola hybrids with the Pioneer Protector *S. sclerotiorum* resistance are reported to provide moderate genetic resistance that can be used as a component of integrated disease management (https://intelseed.ca/uploads/Sclerotinia_Stem_Rot_of_Canola-2012.pdf). The nonavailability of plants with stable and complete resistance against *S. sclerotiorum* indicates the need for more directed approaches to develop new and possibly better sources of *S. sclerotiorum* resistance. It is possible to improve host resistance through recurrent selection, marker-assisted introgressive breeding, and genome editing or genetic modifications. We sought

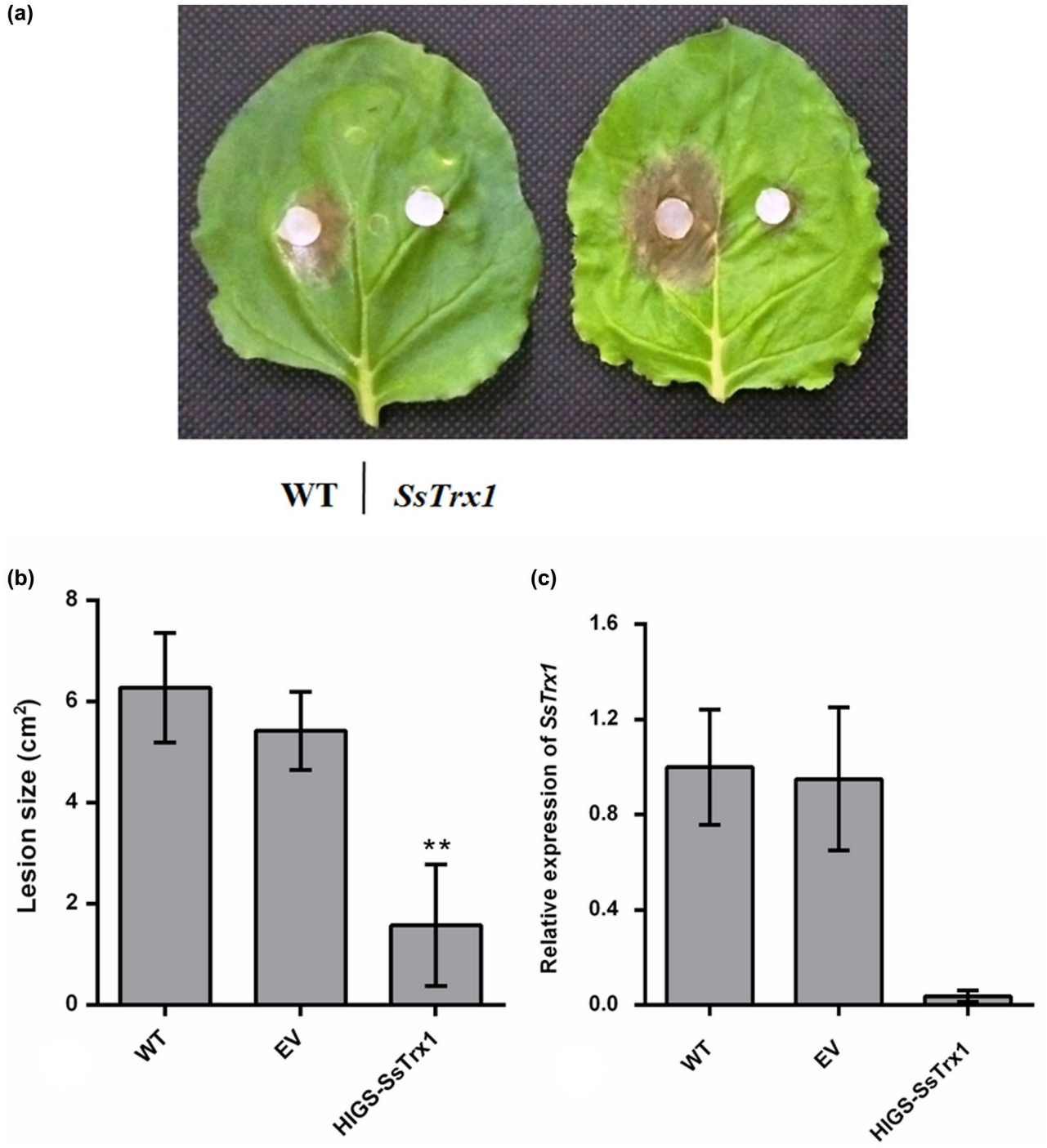

FIGURE 6 The disease resistance analysis of wild type (WT), empty vector (EV), and host-induced gene silencing (HIGS)-*SsTrx1*-*Nicotiana benthamiana* against *Sclerotinia sclerotiorum* using the agro-infiltration method. (a) Pathogenicity assay of WT and HIGS-*SsTrx1*-*N. benthamiana* at 72 hr postinoculation (hpi). (b) Lesion size (cm^2) of WT, EV, and HIGS-*SsTrx1*-*N. benthamiana* under in vivo conditions ($n = 20$; $df = 9, 2$; $p < .01$) at 72 hpi. The bars indicate the standard error and asterisks ** denote significant differences (one-way analysis of variance, Tukey post hoc test). (c) Relative gene expression of *SsTrx1* in HIGS-*SsTrx1*-*N. benthamiana* compared with WT and EV plants

to create a novel source of resistance by selective editing of plant genes or by host-induced suppression of pathogenicity or virulence factors in the pathogen.

RNAi-based gene silencing allowed functional characterization of the *SsTrx1* gene and investigation of HIGS to manage *S. sclerotiorum* in *A. thaliana* and *N. benthamiana*. This was evident from the reduced expression of this gene in *S. sclerotiorum* transformants (*SsTrx1-01*, *SsTrx1-02*, and *SsTrx1-03*). These transformants also produced low virulence following inoculation on detached leaves of *A. thaliana* and *N. benthamiana*. Similar results were obtained following the inoculation of detached leaves and stems of *B. napus*. In planta investigations were also supported by in vitro studies, which showed

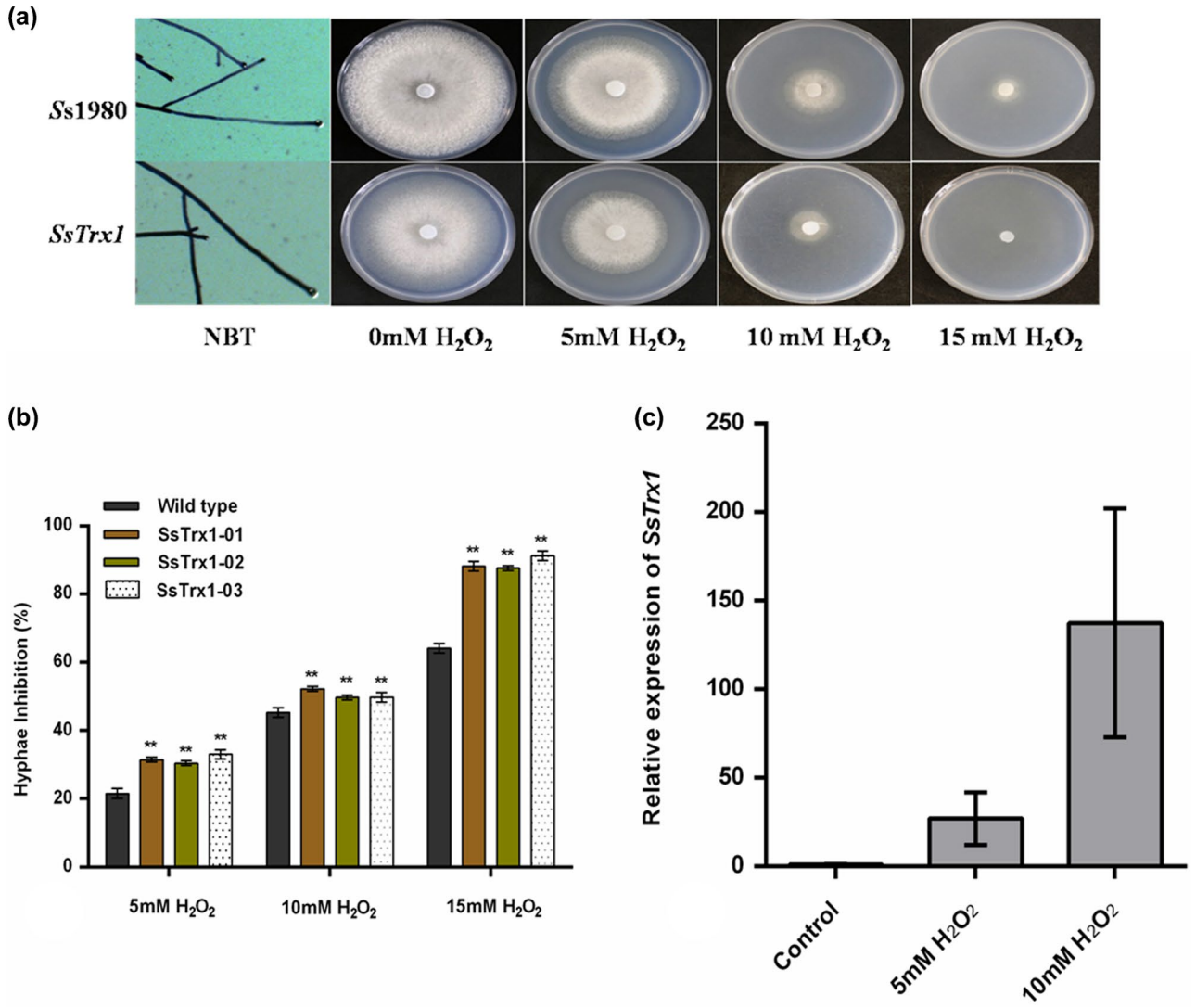

FIGURE 7 The histological examination of hyphal growth and inhibition rate between *Sclerotinia sclerotiorum* strain 1980 and *SsTrx1-01*, *SsTrx1-02*, and *SsTrx1-03* transformants in potato dextrose agar (PDA) supplemented with different H_2O_2 concentrations. (a) Nitroblue tetrazolium stained hyphae at 2 days postinoculation and hyphal growth at 0, 5, 10, and 15 mM H_2O_2 between *S. sclerotiorum* strain 1980 and *SsTrx1-01* at 72 hr postinoculation (hpi). (b) The inhibition rate in PDA with 5, 10, and 15 mM H_2O_2 concentrations at 12, 24, 36, 48, 60, and 72 hpi. The bars indicate the standard error and asterisks (**) denote significant differences ($p < .01$, one-way analysis of variance). (c) Relative gene expression of *SsTrx1* in hyphae treated with 10 mM H_2O_2 as compared with 5 mM H_2O_2 and control

significant reductions in mycelium growth rates and sclerotial production following PDA inoculations with *SsTrx1* transformants compared to inoculation with the WT strain. Mycelial morphology was also adversely impacted. These experiments clearly demonstrated that normal expression levels of *SsTrx1* are essential for the progression of infection and sclerotial development. The importance of the Trx system in maintaining redox balance in the pathogen has been emphasized earlier (Zhang et al., 2019).

HIGS is an RNAi technology where small RNAs made in the plant can silence specific genes of the pathogen. These small RNAs are produced through double-stranded RNA in transgenic plants and this technology has been used to create host resistance (Andrade et al., 2015; Derbyshire et al., 2019; Hu et al., 2015; Hua et al., 2018). HIGS is an efficient and sequence-specific technology that is a reversible and environmental-friendly approach to analyse gene function. Induced mutagenesis and CRISPR/Cas9 can be used for the same purpose, but outcomes of induced mutagenesis are random, while those of CRISPR/Cas9 are irreversible and complete gene knockouts are produced (Mao et al., 2019; Tuo et al., 2019; Wu et al., 2020). The only limitation of RNAi technologies is the likely binding of small interfering RNA to off-target genes showing high sequence homology with the gene of interest (Lundgren & Duan, 2013). Off-target binding can alter the experimental outcomes if it occurs in nontarget genes. Off-target binding at other sites of the genome of interest may have no impact. The present study had the advantage that the selected gene (*SsTrx1*) is relatively unique. Multiple sequence alignment and phylogenetic tree construction showed it to be different from corresponding genes from other plant species or fungal species in terms of overall homology and query coverage. *SsTrx1* was also distinct from the previously reported *S.*

sclerotiorum TrrR1 gene (Zhang et al., 2019). Apparent distinctness of this gene allowed us to exploit the sequence-dependent nature of RNAi to design double-stranded RNA sequences to avoid binding to off-target sites in *A. thaliana* and *N. benthamiana*. HIGS-*A* and HIGS-*N* systems developed during the current study were able to reduce *SsTrx1* expression. Down-regulation of the gene also resulted in reduced pathogenicity and virulence of the pathogen. Only small lesions were produced on detached leaves of *A. thaliana* and *N. benthamiana* following infection with a WT strain of *S. sclerotiorum*. In contrast, WT plants and transformants with EV were highly susceptible. *A. thaliana* lines HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48* showed high H_2O_2 and O_2^- accumulation, thereby confirming the role of *SsTrx1* in ROS detoxification to protect against oxidative stress imposed by the host plant.

In conclusion, we report that the *SsTrx1* gene in *S. sclerotiorum* aids pathogenicity, virulence, and sclerotial development. Our studies also suggested a key role of *SsTrx1* in the antioxidant defence pathway to detoxify H_2O_2 and O_2^-. HIGS of the *SsTrx1* gene can be used to engineer resistance against *S. sclerotiorum* in field crops.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials and fungal strain

A. thaliana ecotype Col-0 and an undesignated accession of *N. benthamiana* plants were used for transformation experiments. Plants were raised in pots containing autoclaved soil mix (Pindstrup) and maintained in growth chambers at 20 ± 4 °C with a 16/8-hr light/dark cycle and a relative humidity of 70% for about 5 weeks. The leaves of *B. napus* 'Zhongshuang11' were used for seedling stage virulence assays. The WT strain of *S. sclerotiorum* 1980 was used for transformation and inoculation. The fungal strain was cultured and maintained on PDA (200 g potato infusion, 20 g dextrose, and 20 g agar in 1 L water, pH 5.6 ± 0.2) at 20 °C with a 12/12 hr light/dark cycle (Clarkson et al., 2003; Godoy et al., 1990).

4.2 | RNAi and HIGS vector construction and transformation

We selected *SsTrx1* for our studies as this gene has been shown to be strongly up-regulated in the resistant (R) pool of *Brassica oleracea* leaves and stems inoculated with *S. sclerotiorum* strain 1980 (Ding et al., 2019; Figure S1). Gene-specific primer pairs (Table S1) were designed to amplify the coding sequence of the *SsTrx1* gene from the cDNA library of *S. sclerotiorum* strain 1980 using Primer Premier 5 software (http://www.premierbiosoft.com). A complete gene fragment was amplified and cloned into the pCIT vector (Yu et al., 2012). The sense and antisense fragments were ligated into a pCIT vector following the homologous recombination ligation method (Finnigan & Thorner, 2015; Jacobus & Gross, 2015). The hygromycin resistance gene cassette was also inserted into the pCIT vector (*Xba*I site) to generate the RNAi pCIT-*SsTrx1* vector. The RNAi pCIT-*SsTrx1* vector was then mobilized into *S. sclerotiorum* strain 1980 via a protoplast transformation method as devised by Rollins (2003). The RNAi *S. sclerotiorum* transformants were selected on PDA supplemented with hygromycin.

For HIGS vector construction, the complete RNAi gene cassette sense-intron-antisense from the RNAi pCIT-*SsTrx1* vector was digested and inserted into plant expression vectors pBinGlyRed3 (GenScript) and pC2301M1B (Fu et al., 2017). The pBinGlyRed3 vector carries a gene encoding red fluorescent protein. The plasmids HIGS-pBinGlyRed3-*SsTrx1* and HIGS-pC2301M1B-*SsTrx*1 were then incorporated into *Agrobacterium tumefaciens* GV3101 via separate electroporation events according to the protocol devised by Wise et al. (2006). The HIGS-pBinGlyRed3-*SsTrx1* vector was transformed into *A. thaliana* (Col-0) plants at the flowering stage using the *Agrobacterium*-mediated floral-dip transformation protocol by Zhang et al. (2006). The HIGS-pC2301M1B-*SsTrx*1 vector was transformed into *N. benthamiana* using the agro-infiltration method as described by Li (2011). The recombinant culture was infiltrated into the underside of leaves of 1–2-month-old plantlets using a plastic syringe with a blunt tip. After infiltration, the plants were kept in the dark for 72 hr to induce transient gene expression. At 72 hr post-agro-infiltration, the plants were inoculated with *S. sclerotiorum* strain 1980.

4.3 | Screening of transformed plants and progeny analysis

The transformed *A. thaliana* plants were allowed to grow for 4–5 weeks in growth chambers under controlled conditions. The self-pollinated seeds were harvested and analysed by the red fluorescence screening method (Ali et al., 2012; Stuitje et al., 2003). The red-coloured seeds were sown and harvested. The same procedure was repeated until the T_3 generation. Segregation ratios were recorded among T_2 seeds and only the progenies consistent with a 3:1 Mendelian segregation ratio were retained for further analysis. Statistical analysis including the Tukey post hoc test was conducted using SAS software (SAS Institute Inc. [SAS], 2013). PCR amplification was carried out using gene-specific primers SsTrx1nF/SsTrx1nR for screening of transformed plants (Table S1).

4.4 | Inoculation and pathogenicity evaluation

For virulence analysis, the detached leaves of *A. thaliana*, *B. napus*, and *N. benthamiana* and detached stems of *B. napus* were inoculated with *S. sclerotiorum* strain 1980 and RNAi-transformed strains of *S. sclerotiorum*, *SsTrx1-01*, *SsTrx1-02*, and *SsTrx1-03*, using a standard inoculation technique (Mei et al., 2015; Taylor et al., 2018). The expanded leaves and stems were excised and inoculated with actively growing mycelial agar plugs (1 mm diameter for *A. thaliana* leaves

and 6 mm diameter for *B. napus* leaves and stems and *N. benthamiana* leaves). Nine leaves of *A. thaliana*, 20 leaves and 10 stems of *B. napus*, and 10 leaves of *N. benthamiana* were inoculated in each replication. The experiments were replicated three times. The inoculated leaves and stems were incubated at 22 °C with 95%–100% relative humidity. The lesion size was noted and photographed at 24 hpi (*A. thaliana* leaves) or 48 hpi (*B. napus* leaves and stems and *N. benthamiana* leaves). For HIGS experiments, transformed *N. benthamiana* plants were inoculated with *S. sclerotiorum* strain 1980, at 72 hr after agro-infiltration. Inoculated plants were first kept in the dark for 24 hr and then shifted to illuminated conditions for 48 hr at 20 ± 4 °C. Ten leaves from each transformed plant along with controls were inoculated into two replications. Lesion size was recorded at 72 hpi.

In the case of *A. thaliana* transformants (generations T_1–T_3), detached leaves (in vitro) and intact leaves in potted plants (in vivo) were inoculated with *S. sclerotiorum* strain 1980. *A. thaliana* WT Col-0 and EV plants were used as controls. Five leaves per transformed line along with control plants were inoculated in each replication. The experiment was repeated twice. Lesion size was recorded at 24 hpi. The mean lesion size (cm^2) and standard error were calculated using SAS software (SAS Institute Inc. [SAS], 2013). The analysis largely involved the use of analysis of variance to establish the significance of differences between the groups. The Tukey post hoc test was used to determine the difference of each group compared to the control.

4.5 | Morphological characterization of *S. sclerotiorum* RNAi transformants

The mycelium growth rates (cm) of WT and *SsTrx1-01* were measured at 12, 24, 36, 48, 60, and 72 hpi in three replications. Mycelial morphology was examined at 1, 2, 3, 5, 7, 21, and 25 dpi in three replications. The average number of sclerotia produced per plate along with their dry weight (g) was measured at 25 dpi in three replications. Statistical analysis including the Tukey post hoc test was performed using SAS software (SAS Institute Inc. [SAS], 2013).

4.6 | RNA extraction and RT-qPCR

The relative gene expression level of *SsTrx1* was assayed with a CFX96 Real-Time RT-qPCR System (Bio-Rad) at various stages of *S. sclerotiorum* development. Total RNA was harvested from young mycelium of *S. sclerotiorum* strain 1980 at 1, 2, 3, 5, 6, and 7 dpi using the TRIzol method (TianGen) (Rio et al., 2010). RNA was also extracted from the mycelium of *S. sclerotiorum* RNAi transformants at 3 dpi to assess the relative *SsTrx1* gene expression level in *S. sclerotiorum* transformants.

To analyse the interference with *SsTrx1* expression, RNA was harvested from WT, EV, HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48* transgenic *A. thaliana* plants inoculated with *S. sclerotiorum* strain 1980 at 24 hpi and from inoculated WT, EV, and *SsTrx1* transgenic *N. benthamiana* plants inoculated with *S. sclerotiorum* 1980 at 48 hpi. To assess *SsTrx1* gene expression under oxidative stress conditions, RNA was harvested from actively growing mycelium of *S. sclerotiorum* strain 1980 grown on PDA supplemented with 5 mM H_2O_2 and 10 mM H_2O_2 at 48 hpi. The RNA was used to synthesize the first-strand cDNA as a template for RT-qPCR according to the specifications of the iScript cDNA Synthesis Kit (Bio-Rad). The *S. sclerotiorum* gene *Tubulin* was used as an internal standard. The experiment was performed in a 10 μl reaction mixture using the iTaq Universal SYBR Green Supermix (Bio-Rad). The quantitative assays were repeated three times for each cDNA sample. The qPCR cycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 56–60 °C for 1 min and melting curve ramping from 65 °C to 95 °C at a rate of 0.5 °C after every cycle. The data were analysed with CFX Manager v. 3.0 and the relative transcript level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Primer sequences used for RT-qPCRs are available in Table S1.

4.7 | Evaluation of H_2O_2 and O_2^- accumulation

S. sclerotiorum strain 1980 and the RNAi transformant *SsTrx1-01* were cultured on PDA plates supplemented with 5, 10, and 15 mM H_2O_2 to analyse the effects of oxidative stress. The mycelium growth inhibition rates were calculated at 12, 24, 36, 48, 60, and 72 hpi. The experiment was repeated twice in three replications for each concentration of H_2O_2. Statistical analysis was conducted using SAS software (SAS Institute Inc. [SAS], 2013). For qualitative analysis of O_2^- accumulation, the actively growing hyphae (at 2 dpi) of *S. sclerotiorum* strain 1980 and *SsTrx1-01* RNAi transformants were stained with NBT for 5 hr followed by washing with distilled water. The stained hyphae were then examined under the microscope (Eclipse Ci-L; Nikon) and photographed (Kumar et al., 2014). Superoxide (O_2^-) and H_2O_2 accumulation was measured after inoculation with *S. sclerotiorum* strain 1980 from HIGS-*Trx1-10*, HIGS-*Trx1-19*, and HIGS-*Trx1-48 A. thaliana* transgenic leaves along with controls: WT (untransformed *A. thaliana*) and CK (untransformed *A. thaliana* without inoculation). The mycelial agar plugs were removed from leaves at 0, 6, and 12 hpi and dipped in Falcon tubes containing DAB or NBT staining solution. The tubes were then incubated in the dark for 5 hr. The staining solution was subsequently replaced with a bleaching solution (ethanol, acetic acid, and glycerol at a ratio of 3:1:1). The tubes were then kept in a boiling water bath (c.95 °C) for 15 min. The leaves were photographed. The experiment was repeated twice for each of the three replications.

ACKNOWLEDGEMENTS

This research was financially supported by the National Science Foundation of China (NSFC; 31801395 and 31971978), the Natural Science Foundation of Chongqing (cstc2019jcyj-zdxmX0012 and cstc2019jcyj-msxmX0486), and the Fundamental Research Funds for the Central Universities (XDJK2018AA004, XDJK2018B022,

and SWU120075). The authors declare no conflict of interest. We thank reviewers for their kind suggestions.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Rana, K., Ding, Y., Banga, S.S., Liao, H., Zhao, S., Yu, Y.&et al. (2021) *Sclerotinia sclerotiorum Thioredoxin1 (SsTrx1)* is required for pathogenicity and oxidative stress tolerance. *Molecular Plant Pathology*, 22, 1413–1426. https://doi.org/10.1111/mpp.13127