Host-Induced Gene Silencing of a Multifunction Gene Sscnd1 Enhances Plant Resistance Against Sclerotinia sclerotiorum

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Sclerotinia sclerotiorum is a devastating necrotrophic fungal pathogen and has a substantial economic impact on crop production worldwide. Magnaporthe appressoria-specific (MAS) proteins have been suggested to be involved in the appressorium formation in Magnaporthe oryzae. Sscnd1, an MAS homolog gene, is highly induced at the early infection stage of S. sclerotiorum. Knock-down the expression of Sscnd1 gene severely reduced the virulence of S. sclerotiorum on intact rapeseed leaves, and their virulence was partially restored on wounded leaves. The Sscnd1 gene-silenced strains exhibited a defect in compound appressorium formation and cell integrity. The instantaneous silencing of Sscnd1 by tobacco rattle virus (TRV)-mediated host-induced gene silencing (HIGS) resulted in a significant reduction in disease development in tobacco. Three transgenic HIGS Arabidopsis lines displayed high levels of resistance to S. sclerotiorum and decreased Sscnd1 expression. Production of specific Sscnd1 siRNA in transgenic HIGS Arabidopsis lines was confirmed by stem-loop qRT-PCR. This study revealed that the compound appressorium-related gene Sscnd1 is required for cell integrity and full virulence in S. sclerotiorum and that Sclerotinia stem rot caused by S. sclerotiorum often causes significant losses in crop production.

Keywords: compound appressorium, pathogenicity, host-induced gene silencing, Sclerotinia sclerotiorum, Sscnd1

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a well-known necrotrophic phytopathogenic fungus with a broad host range, including many economically important crops, such as oilseed rape (Brassica napus), sunflowers, soybeans, peanuts and lentils (Boland and Hall, 1994; Bolton et al., 2006). Sclerotinia stem rot caused by S. sclerotiorum often causes significant losses in crop production.

As a necrotrophic parasite, S. sclerotiorum has evolved a sophisticated infection process to effectively infect hosts (Kabbage et al., 2015). To adhere to the host surface, the tips of its hyphae become swollen and extensive branch prior to penetration and then develop a multicellular, melanin-rich hyphal penetration structure, called compound appressorium (Jamaux et al., 2007; Huang et al., 2008; Uloth et al., 2016). This formation of compound appressorium is essential for the process in which fungi penetrate the host cuticle and form infectious hyphae to spread horizontally beneath the host cuticle (Liang and Rollins, 2018).
Multiple *S. sclerotiorum* genes are required for the formation and development of compound appressorium. The disruption of GATA-type transcription factors (SsAMS2 and SsNSD1) impairs compound appressorium formation and virulence (Li et al., 2017; Liu et al., 2018). The secretory proteins SsCaf1 and SsRh1 are highly induced in the initial infection stage, and their gene-silenced strains exhibit defects compound appressorium formation (Xiao et al., 2014; Yu et al., 2017). Additionally, oxalic acid (OA) accumulation, the cAMP-PKA signaling pathway and other genes, such as the genes γ-glutamyl transpeptidase (Ssgt1) and survival factor 1 (Ssvf1), have also been shown to be associated with the development of compound appressorium (Jurick and Rollins, 2007; Li et al., 2012; Liang et al., 2015a,b; Yu et al., 2019). Similar to *S. sclerotiorum*, *Magnaporthe oryzae* also produces appressoria to penetrate host plant cells and initiate infection (Dean, 1997; Hamer and Talbot, 1998; Zhang et al., 2016a). GAS1 and GAS2, encoding the Magnaporthe appressoria-specific (MAS) proteins, function in the appressorium formation and fungal virulence in *M. oryzae* (Xu and Hamer, 1996; Xue et al., 2002). BcGAS2, a homolog gene of the *M. oryzae* GAS2, is required for appressorial function but is not essential for the growth and infection of *Botrytis cinerea* (Schamber et al., 2010). Although two MAS homologs are overexpressed in the infection cushion of *S. sclerotiorum* (Sexton et al., 2006), there is no experimental evidence of their functions.

RNA interference (RNAi) is a universal gene regulation mechanism in eukaryotes that involves exogenous double-stranded RNA (dsRNA) (Raulcombe, 2005). RNA-silencing technology has been exploited extensively to knock down fungal genes to improve resistance in plants (Duan et al., 2012). Interestingly, some insect pests and nematodes can be successfully controlled by merely feeding dsRNAs (RNAi constructs) of their genes (Huang et al., 2006; Baum et al., 2007; Huvinne and Smagge, 2010). Recently, this strategy, named host-induced gene silencing (HIGS), has been applied to reduce pathogen aggressiveness, such as *Puccinia* (Panwar et al., 2013; Yin et al., 2015; Zhu et al., 2017; Qi et al., 2018) and *Fusarium* (Ghag et al., 2014; Cheng et al., 2015; Chen et al., 2016) in wheat, *Rhizoctonia solani* in tall fescue (Zhou et al., 2016), *Phytophthora infestans* in potato (Jahan et al., 2015; Sanju et al., 2015), *Bremia lactucae* in lettuce (Govindarajulu et al., 2015) and *Verticillium* in *Arabidopsis*, tomato and cotton (Zhang et al., 2016b; Song and Thomma, 2018; Xu et al., 2018).

Thus, the blockage of compound appressorium differentiation by interfering with the *S. sclerotiorum* MAS genes is an efficient strategy for decreasing the disease phenotype. In this study, Sscnd1 encoding a MAS homolog, was characterized in *S. sclerotiorum*. The function of Sscnd1 in the compound appressorium formation and pathogenicity of *S. sclerotiorum* was determined. We further explored the potential of improving plant resistance via target silencing of Sscnd1 by gene silencing. Our data suggest that Sscnd1 is required for compound appressorium formation, cell integrity and full virulence in *S. sclerotiorum*.

**MATERIALS AND METHODS**

**Fungal Strains, Plants, and Culture Conditions**

The *S. sclerotiorum* isolate 1980 (Godoy et al., 1990) was used as the wild-type strain and cultured on potato dextrose agar (PDA) (Difco Laboratories, Detroit). Sscnd1 gene-silenced strains were cultured on PDA supplemented with hygromycin B at 100 µg/mL (Calbiochem, San Diego, CA). The wild-type *Arabidopsis thaliana* Col-0 (Columbia zero background ecotype) and its transgenic lines were grown in a controlled environment chamber with 16-h/23°C days and 8-h/16°C nights at 100 µmol/m²/s light intensity.

**Bioinformatic Analysis of Sscnd1**

The sequences of Sscnd1 were obtained from the genomic sequence database of *S. sclerotiorum* genome (http://fungidb.org/common/downloads/Current_Release/Sclerotiorum/). BlastP analysis was performed on the website of NCBI (http://www.ncbi.nlm.nih.gov/). The signal peptide sequence and transmembrane domain were predicted using SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/), TMHMM 2.0 (http://www.ch.embnet.org/software/TMPRED_form.html). Multiple sequence alignment was implemented with DNAMAN6.0 (Lynnon BioSoft, Quebec, Canada) and CLUSTALX2.0 (Chenna et al., 2003). The phylogenetic tree was constructed with MEGA 6.0 software (Tamura et al., 2013) using the maximum likelihood method, and the bootstrap test was replicated 1,000 times.

**RNA Extraction, cDNA Synthesis and qRT-PCR**

To evaluate the Sscnd1 expression levels during hyphal development, the wild-type strain was cultured on cellophane over PDA, and mycelia were harvested at 1 and 2 days post-inoculation (dpi) (hyphae), 3 and 4 dpi (initial sclerotia), 5, 6, and 7 dpi (developing sclerotia), and 8 dpi (mature sclerotia). To examine the Sscnd1 expression levels during infection stages, the wild-type strain was cultured in potato dextrose broth (PDB) for 2 days and the mycelia were harvested and ground into fragments. The hyphal fragments were suspended in ddH₂O and then sprayed on the leaves of rapeseed, as well as on the cellophane placed on PDA plates as controls. The inoculated leaves and hyphae growing on PDA plates were harvested at 0, 3, 6, 9, 12, 24, and 48 h post inoculation (hpi). Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA), and first-strand cDNA was synthesized for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR was performed using Bio-Rad CFX96 Real-Time System (America) and Quantitect SYBR Green PCR master mix (Bio-Rad, USA) according to the manufacturer’s instructions. The β-tubulin gene *Sstub1* (SS1G_04652) was used as the internal reference for normalization. The transcript level of the gene of interest was calculated from the threshold cycle using
the $2^{-\Delta \Delta CT}$ method (Livak and Schmittgen, 2001) with three replicates, and the data were analyzed using CFX Manager™ v3.0. The primers were listed in Supplementary Table 1.

**Binary Constructs**

The Sscnd1 gene-silencing vector was constructed based on the plasmid pCIT (Yu et al., 2017). The sense and antisense fragments of Sscnd1 with a length of 418 bp were cloned into the corresponding clone sites of pCIT, and the hygromycin resistance gene cassette from pSKH (Hamid et al., 2013) was subsequently inserted into it. The resulting Sscnd1-RNAi construct pSscnd1 was transformed into the S. sclerotiorum wild-type strain 1980 according to the method of Rollins (2003). Meanwhile, the plasmid pCIT containing a hygromycin resistance gene cassette was used as the empty vector pRNAi. The strain containing the empty vector pRNAi was used as the control in whole experiments.

The sense and antisense fragments of Sscnd1 were cloned and flanked with the malate synthase gene intron 3 from A. thaliana (i3). The cassette was cloned into the plasmid pBinGlyRed3, which contained a red fluorescent protein (DS Red). The resulting HIGS construct HIGS-Sscnd1 was introduced into the Agrobacterium tumefaciens strain GV3101 by electroporation (Wise et al., 2006) and then transformed into A. thaliana Col-0 using the floral dip method (Clough and Bent, 1998).

**Pathogenicity Assays**

The pathogenicity of the S. sclerotiorum wild-type, empty vector, and Sscnd1 gene-silenced strains was evaluated in the unwounded and wounded (wounded with a dissecting needle) leaves of B. napus (Zhongshuang 11). The 0.6-cm mycelium-colonized agar plugs obtained from actively growing colony edges were used to inoculate onto the leaves. The inoculated leaves were kept in 90% relative humidity at 20°C. The lesions were measured at 48 hpi. Each strain was evaluated with three leaves in one replicate and the experiments were performed five times.

The 4-week-old HIGS-Sscnd1 transgenic A. thaliana lines were inoculated with 0.2-cm mycelium-colonized agar plugs of the S. sclerotiorum wild-type strain 1980 from actively growing colony edges. Lesion area was measured at 24 hpi for in vitro inoculation and 4 dpi for in vivo inoculation. The experiments were performed at least five times with five leaves or plants for every line in one replicate.

To evaluate the resistance of HIGS-Sscnd1 transgenic A. thaliana lines to B. cinerea, the 0.2-cm mycelium-colonized agar plugs of B. cinerea strain B05.10 from actively growing colony edges were used to inoculate the detached leaves of HIGS-Sscnd1 transgenic A. thaliana lines. Lesion area was measured at 24 hpi. The experiments were performed at least five times with five leaves for every strain in one replicate.

The lesion area ($S$, cm$^2$) was calculated with the formula $S = \pi ab/4$, where $a$ and $b$ represent the long and short diameter of an approximately elliptical lesion.

**Detection of siRNA in HIGS-Sscnd1 Transgenic A. thaliana Lines**

To detect Sscnd1 siRNA production in HIGS-Sscnd1 transgenic A. thaliana lines, stem-loop qRT-PCR was performed against the Sscnd1 gene of S. sclerotiorum according to Mahto et al. (2020) with some modifications. For this purpose, a putative siRNA sequence (UAACUUGAGGAAGAGUUUCAC) was identified within the 418 bp Sscnd1 sequence employed for the construction of RNAi vector via siDirect version 2.0 (http://sidirect2.mai.jp/), which is functionally appropriate for knocking down the Sscnd1 gene expression. Low molecular weight RNA was isolated and then utilized to synthesize cDNA using stem-loop primer (ST-Sscnd1). Subsequently, stem-loop qRT-PCR was performed using siRNA specific primers. The primers were listed in Supplementary Table 1. qRT-PCR was performed using Bio-Rad CFX96 Real-Time System (America) and Quantitect SYBR Green PCR master mix (Bio-Rad, USA) according to the manufacturer's instructions. The A. thaliana U6 gene AtU6-26 was used as the internal reference for normalization. The transcript level of the gene of interest was calculated from the threshold cycle using the $2^{-\Delta \Delta CT}$ method (Livak and Schmittgen, 2001) with three replicates, and the data were analyzed using CFX Manager™ v3.0.

**High Osmotic Stress Assay**

To calculate the inhibition rate of hyphal growth when cultured with high osmotic stress and membrane damage stress, the 0.6-cm mycelium-colonized agar plugs of Sscnd1 gene-silenced strains, wild-type and empty vector strain obtained from actively growing colony edges were inoculated on the center of PDA plates supplemented with 2% sorbose, 5% sorbose, 1 M sorbitol, 1.2 M sucrose and 0.02% sodium dodecylsulphate (SDS), respectively. Colony radius was measured every 12 h before the colony reached margins of the plates. The colony phenotype photographs were taken at 3 dpi. Each experiment was repeated three times with five plates for every treatment in one replicate.

**Compound Appressorium Assay**

Compound appressorium formation of Sscnd1 gene-silenced strains was observed according to Yu et al. (2019). The 0.6-cm mycelial plugs were inoculated onto parafilm-overlaid PDA plates and rapeseed leaves. The plugs were removed at 8 hpi. the parafilm surface was stained with 5% trypan blue. The compound appressorium on parafilm was observed using a microscope. The inoculated rapeseed leaves were stained with 5% trypan blue for 12 h and then cleared with ethanol/acetic acid (3:1 v/v) solution for 12 h. The compound appressorium on rapeseed leaves were observed using an electron microscope (JEOL JEM-6390LV). The experiment was repeated for three independent times.

**TRV-Based Sscnd1 Gene Silencing in N. benthamiana**

To determine the role of Sscnd1 during infection, a TRV-based gene-silencing system was applied in N. benthamiana. A 218-bp fragment of Sscnd1 named VIGS-Sscnd1-1 and a 296-bp fragment of Sscnd1 named VIGS-Sscnd1-2 were amplified with primers Sscnd1-VIGS-1F/R and Sscnd1-VIGS-2F/R, respectively.
The amplicons were inserted into TRV2 vector (Liu and Page, 2008) to produce the VIGS constructs TRV2:: Sscnd1-1 and TRV2:: Sscnd1-2. The recombinant virus TRV2:: GFP was applied as a control. The infiltration of N. benthamiana plants was performed according to Liu and Page (2008). The upper leaves from the infiltrated N. benthamiana leaf were inoculated with S. sclerotiorum wild-type strain 1980 seven days after infiltration. The experiment was repeated for five independent times and every construct was infiltrated with at least five plants in one replicate. Lesion phenotypes were recorded at 48 hpi.

**RNA Sequencing and Data Analysis**

The mycelia of wild-type stain 1980 and Sscnd1 gene-silenced strains (Sicnd1-9 and Sicnd1-20) on the PDA plate were collected at 48 hpi. The sequencing library of three samples with two replicates was generated using the Illumina RNA Library Prep Kit (NEB, USA) following the manufacturer's recommendation, and sequenced on an Illumina Hiseq 2000 platform that yields 100-bp paired-end reads. The raw reads were filtered to obtain high-quality clean reads by removing adaptor sequences, duplicated sequences, reads containing more than 5% “N” (i.e., ambiguous bases in reads), and reads in which more than 50% of the bases showed a Q-value (i.e., Bonferroni-adjusted P-value) ≤ 5. Clean reads were aligned to the reference genome of S. sclerotiorum (http://fungidb.org/common/downloads/Current_Release/Sscerotiorum) by using the TopHat2 (http://ccb.jhu.edu/software/tophat/index.shtml) with default parameters except that the Q value was set to 100. Gene expression was quantified using Salmon (https://combine-lab.github.io/salmon/). The raw counts were
normalized by TPM (Transcripts Per Million reads) and the differential expression analysis was performed using the DEseq2 (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html). The threshold determining the significance of differentially expressed genes (DEGs) among multiple tests was set at a p-adjust < 0.05 and \(|\log2 \text{ ratio}| \geq 1\) (Mao et al., 2018). GO enrichment analyses were analyzed on the free online platform of Majorbio Cloud Platform (www.majorbio.com).

RESULTS

Identification and Expression Patterns of the Genes Encoding MAS Proteins in S. sclerotiorum

A total of five MAS homologs were identified in the genome of S. sclerotiorum (Figure 1). Among them, SS1G_11468 had the highest expression level during the infection of both Brassica oleracea leaves and stems, as revealed by RNA-seq in the previous study (Mei et al., 2016; Ding et al., 2019) (Supplementary Figures 1A,B). Additionally, SS1G_11468 was the most highly expressed gene during infecting Brassica oleracea leaves (Supplementary Figure 1C). Furthermore, the sequence of SS1G_11468 showed best matches to a specific expressed sequence tag (EST) DV643832 from the infection cushion library of S. sclerotiorum. SS1G_11468 contains a 747-bp ORF with two exons and encodes a protein with a length of 248 amino acids. The N-terminus of SS1G_11468 was predicted to contain a typical signal peptide with SignalP 5.0 Server and an extracellular and non-membrane location with TMHMM 2.0 or TMpred. The predicted cleavage site was between amino acid positions 18 and 19. Sequence comparison and phylogenetic tree analysis showed that SS1G_11468 exhibited high sequence similarity with B. cinerea CND1 (BC1G_08931) (86.29% identity in amino acid sequence, E-value: 7e-122) and M. oryzae MAS3 (MGG_11595) (58.20% identity in amino acid sequence, E-value: 6e-074) (Figures 1A,B). Therefore, SS1G_11468 was named Sscnd1.

The expression patterns of Sscnd1 during the different developmental stages and infection processes were determined via qRT-PCR. The results showed that Sscnd1 was highly expressed during the hyphal growth stage (Figure 2A). When inoculated on B. napus leaves, Sscnd1 expression dramatically increased from 3 hpi to 12 hpi by almost 39-fold (Figure 2B). However, the other four homologous MAS-related genes showed significantly lower expression than Sscnd1 during infection processes (Supplementary Figure 2). These results suggest that Sscnd1 is strongly induced during the infection of S. sclerotiorum, especially at the initial infection stage.

Sscnd1 Gene-Silenced Strains Showed Impaired Mycelial Growth

To determine the possible roles of Sscnd1 in the development of the S. sclerotiorum mycelium, Sscnd1-knockdown strains were obtained via RNAi. The RNAi vector pSicnd1 was transformed into the S. sclerotiorum wild-type strain 1980 (Figure 3A). Two strains, Sicnd1-9 and Sicnd1-20, with reduced Sscnd1 expression levels were chosen (Figure 3B). Multiple sequence alignment showed that the amplified fragment of Sscnd1 exhibited low similarity with the other four S. sclerotiorum MAS-related genes in nucleic acid sequence (SS1G_10311: 1.20% identity, SS1G_14237: 1.40% identity, SS1G_00173: 2.20% identity, SS1G_00173: 1.80% identity) (Supplementary Figure 3A). We found that the expression of the other four MAS-related genes was unimpaired in Sicnd1-9 and Sicnd1-20 (Supplementary Figure 3B).

Morphological analysis showed that both Sicnd1-9 and Sicnd1-20 exhibited frequent cytoplasmic bleeding at hyphal tips by microscopy (Figure 3C). The proportion of hyphal tips with cytoplasmic bleeding for Sicnd1-9 and Sicnd1-20 was 34.02 and 30.37%, respectively, which was higher than the wild-type strain (2.38%) and control strain (1.59%). In addition to the aberrant morphology of hyphal tips, Sicnd1-9 and Sicnd1-20 exhibited...
significantly reduced mycelial growth and sclerotia formation on PDA plates (Figure 3C). The growth rate was 1.23 cm/12 hpi for the wild-type strain and 1.20 cm/12 hpi for the empty vector strain, but 1.03 cm/12 hpi and 1.10 cm/12 hpi for Sicnd1-9 and Sicnd1-20, respectively (Figure 3D). The results indicate that Sicnd1 is associated with mycelial growth in S. sclerotiorum.

**Sicnd1** Is Required for Compound Appressorium Formation and Full Virulence in S. sclerotiorum

To explore whether Sicnd1 was involved in compound appressorium development, the wild-type, empty vector strain and Sicnd1 gene-silenced strains were inoculated on parafilm-overlayed PDA and on the leaves of B. napus. We found that both Sicnd1-9 and Sicnd1-20 produced fewer compound appressoria on parafilm or rapeseed leaves than the wild-type strain and empty vector strain (Figure 3E). Additionally, the number of compound appressorium was positively related to the expression of Sicnd1 ($r = 0.920$, $P < 0.05$) (Supplementary Figure 4), indicating that Sicnd1 is associated with compound appressorium formation in S. sclerotiorum.

Furthermore, to examine whether Sicnd1 is involved in the pathogenicity of S. sclerotiorum, the detached B. napus leaves were inoculated with agar plugs derived from the wild-type, empty vector strain and Sicnd1 gene-silenced strains, and we...
found that the lesion areas of *B. napus* leaves infected with Sicnd1-9 and Sicnd1-20 were reduced to 0.52- and 0.58-fold of the wild-type strain and to 0.53- and 0.59-fold of the empty vector strain, respectively (Figures 4A,B). These results indicate that the infection capacity of the Sicnd1 gene-silenced strains is highly impaired. We further monitored the rescue of penetration events by inoculation on the wounded leaves of *B. napus*. We found that Sicnd1 gene-silenced strains induced larger lesions on the wounded leaves than on the intact leaves, but the lesion area was still significantly smaller than wild-type and empty vector strain (Figures 4A,B). These results suggest that Sicnd1 may contribute toward full virulence at the penetration phase of *S. sclerotiorum*.

**Sicnd1 Is in Association With Cell Integrity of *S. sclerotiorum***

To further investigate the role of Sicnd1 in the growth and virulence of *S. sclerotiorum*, we performed whole genome expression profiling analysis of the hyphae in wild-type and Sicnd1 gene-silenced strains through RNA sequencing (RNA-Seq). The raw data were stored in NCBI BioProject database.
FIGURE 6 | The growth of Sscnd1 gene-silenced strains in the presence of various stressors. Phenotypes (A) and growth inhibition (B) of the wild-type strain (WT), empty vector strain (EV), Sicnd1-9 and Sicnd1-20 grown on PDA supplemented with 0.02% SDS, 2% sorbose, 5% sorbose, 1M sorbitol, and 1.2M sucrose, respectively. Photographs were taken at 3 dpi (days post inoculation). One representative biological replicate is shown. Error bars indicate the standard deviation for three replicates. Differences in every stressor group were assessed using Tukey’s HSD test. Different letters indicate statistical significance at the 0.05 level (P < 0.05).

with the accession ID PRJNA744751. Gene expression of Sscnd1 in the silenced strains was reduced to 0.3 fold of wild-type strain, the other four MAS-related genes showed no significant changes (Sicnd1 [Sicnd1-9 and Sicnd1-20]_vs_WT) (Supplementary Figure 5). Additionally, there were 662 differentially expressed genes (DEGs) (Sicnd1 [Sicnd1-9 and Sicnd1-20]_vs_WT), consisting of 269 up-regulated DEGs and 393 down-regulated DEGs (Figure 5A and Supplementary Table 2). To validate the data obtained by RNA-seq, we performed qRT-PCR analysis by choosing 15 S. sclerotiorum genes of interest, and found a high consistency of gene expression between qRT-PCR and RNA-seq (r = 0.952, P < 0.01) (Supplementary Figure 6). Gene ontology (GO) enrichment analysis showed that these down-regulated DEGs were categorized into 14 GO terms (q < 0.01). Of these, the most significantly enriched GO terms were associated with the intrinsic component of membrane and integral component of membrane (Figure 5B). These results indicate that Sscnd1 is associated with the cell membrane integrity.

To explore whether the suppression of Sscnd1 affects the cell membrane integrity in S. sclerotiorum. The tolerance to high osmotic stresses was assessed. Growth on PDA plates supplemented with 2% sorbose, 5% sorbose, 1M sorbitol or 1.2 M sucrose among the wild-type, empty vector strain and Sscnd1 gene-silenced strains was investigated. The results showed that the inhibition of hyphal growth was significantly greater in Sscnd1 gene-silenced strains than in the wild-type strain and empty vector strain (Figures 6A,B). Furthermore, in the presence of 0.02% SDS, which damages cell membrane of organisms (Temme et al., 2012), the growth rate of the Sscnd1 gene-silenced strains was almost completely suppressed, while the wild-type strain and empty vector strain could slowly grow, suggesting that the Sscnd1-silenced strains were more sensitive to SDS. These
results indicate that Sscnd1 is involved in the response to high osmotic stresses and cell integrity.

**HIGS of Sscnd1 in the Host Enhances *S. sclerotiorum* Resistance**

Sequence alignment and phylogenetic tree analysis revealed no genes homologous to Sscnd1 in plants, indicating that Sscnd1 could be a target gene for the application of HIGS to control Sclerotinia stem rot disease. A tobacco rattle virus (TRV)-mediated transient silencing of Sscnd1 was performed in *Nicotiana benthamiana*. Seven days after TRV treatment, plants were challenged with the *S. sclerotiorum* wild-type strain 1980. The relative expression of Sscnd1 in TRV:: Sscnd1-1- and TRV:: Sscnd1-2-infected leaves was reduced by 63 and 66%, as determined by qRT-PCR, compared with that in the control leaves (TRV:: GFP), and the lesion area on TRV:: Sscnd1-1- and...
TRV: Sscnd1-2-infected leaves was reduced by 51 and 56% at 48 hpi, respectively, compared with that on the control plants (TRV:: GFP) (Supplementary Figure 7).

To assess whether the resistance against S. sclerotiorum can be improved by expressing an RNAi construct targeting Sscnd1 in stable transgenic plants, we transferred a HIGS vector containing the RNAi cassette of Sscnd1 into wild-type A. thaliana Col-0 (Figure 7A). All the fifty transgenic lines in T1 generation exhibited smaller lesion areas than the wild-type Col-0 and plants carrying empty vector (EV plants, positive control) (Supplementary Table 3). Of which three transgenic HIGS-Sscnd1 lines (HIGS-Sscnd1-25, HIGS-Sscnd1-39, and HIGS-Sscnd1-42) with the smallest lesion area were continuously self-crossed to develop homozygous lines (Figure 7 and Supplementary Table 3). No significant difference was observed in growth between transgenic and control A. thaliana lines (Supplementary Figure 8). The wild-type Col-0, EV plants, and these homozygous lines of three transgenic HIGS-Sscnd1 in T3 generations were challenged with the S. sclerotiorum wild-type strain 1980 in vitro and in vivo. At 24 hpi, the lesion areas on the leaves of HIGS-Sscnd1-25, HIGS-Sscnd1-39, and HIGS-Sscnd1-42 were reduced by 35, 45, and 33% in vitro and 61, 71, and 53% in vivo compared with those on the leaves of the EV plants, respectively (Figures 7B,C). The in vivo lesion areas were significantly correlated with the in vitro lesion areas (r = 0.985, P < 0.05). The expression of Sscnd1 at 9, 12, and 24 hpi was clearly suppressed in these transgenic HIGS-Sscnd1 lines compared with the Col-0 and EV plants (Figure 7D). To verify the production of specific siRNA (Sscnd1-siRNA) in transgenic HIGS-Sscnd1 A. thaliana lines, stem-loop qRT-PCR was carried out with cDNA of leaf tissues (Supplementary Table 1). The results showed that Sscnd1-siRNA was highly expressed in HIGS-Sscnd1 A. thaliana lines, but no expression of Sscnd1-siRNA was detected in Col-0 and EV plants (Supplementary Figure 9). To rule out the effect that expression of HIGS-Sscnd1 construct activates plant defense responses in plants, several defense-related marker genes (AtPR1, AtPR2, AtPR5, and AtPDF1.2) were performed the expression analysis in the HIGS-Sscnd1 lines. There was no significant difference in these genes in HIGS-Sscnd1 lines compared with Col-0 and EV (Supplementary Figure 10A). Additionally, the expression of four Sscnd1 homologous genes showed no significant changes in the inoculated HIGS-Sscnd1 lines (Supplementary Figure 10B).

Considering that the sequence of Sscnd1 exhibits 87.01% identity in nucleic acid sequence with B. cinerea Bccnd1 (BC1G_08931), the HIGS-Sscnd1, Col-0 and EV lines were challenged with B. cinerea B05.10. We found that the lesion areas on HIGS-Sscnd1-25, HIGS-Sscnd1-39, and HIGS-Sscnd1-42 were reduced at 24 hpi compared with those on the Col-0 and EV lines (Supplementary Figures 11A,B). The relative expression of BC1G_08931 in HIGS-Sscnd1 lines decreased at 24 hpi compared with that in the Col-0 and EV lines (Supplementary Figure 11C).

**DISCUSSION**

As multicellular infectious structures, compound appressoria are formed unless penetration occurs directly via stomata and are essential for S. sclerotiorum to successfully penetrate hosts (Uloth et al., 2016; Liang and Rollins, 2018). Sscnd1 coding a protein with 58.20% identity to M. oryzae MAS3. In this study, the expression of Sscnd1 was upregulated in the early infection stage. The Sscnd1 gene-silenced strains showed a drastic reduction in virulence and compound appressorium formation. The virulence of Sscnd1 gene-silenced strains was partially restored on wounded leaves, but still significantly lower than control strains. These findings indicate that Sscnd1 is associated with compound appressorium formation and fungal full virulence at penetration phase in S. sclerotiorum. In B. cinerea and M. oryzae, when the disruption of the genes coding MAS proteins (BC1G_13581 in B. cinerea, MGG_12337 and MGG_04202 in M. oryzae), though the mutants present impaired virulence, there are no significant changes in the hyphae growth (Xue et al., 2002; Schambler et al., 2010). In contrast with B. cinerea and M. oryzae, Sscnd1 gene-silenced strains showed a reduction in the hyphae growth. The protein sequence alignment showed that Sscnd1 coding a protein with 27.84% identity to BC1G_13581, 37.55% identity to MGG_12337, and 29.9% identity to MGG_04202. The sequence specificity may give new functions of Sscnd1. Homologous genes originate from the same ancestral gene, but they may lose their original functions or evolve new functions in the process of evolution (Li et al., 2005). Therefore, the role of Sscnd1 revealed both common and unique properties compared with those of other plant pathogenic fungi.

S. sclerotiorum secretes OA, enzymes, and effector proteins to induce the necrosis of host cells and absorbs nutrients from dying host cells (Amelem et al., 2011; Williams et al., 2011; Bashi et al., 2012; Kabbage et al., 2013; Guyon et al., 2014; Seibabghiry et al., 2017; Yang et al., 2018). However, the cytoplasmic exudate of the dying cells causes osmotic stress, which inhibits fungal survival, germling differentiation and penetration (Kamamura et al., 2002; Skamnioti and Gurr, 2007). The hyphal tips of Sscnd1 gene-silenced strains exhibited frequent cytoplasmic bleeding. Down-regulated expression of genes in modulating component of membrane and high sensitivity to osmotic stress, suggesting that the Sscnd1 gene-silenced strains have a defect in cell integrity. The association of cell integrity and osmotic stress response with the appressorium formation has been shown in many important pathogenic fungi, such as M. oryzae (Jeon et al., 2008; Deng et al., 2019), Colletotrichum fructicola (Liang et al., 2019), Colletotrichum graminicola (Albarouki and Deising, 2013) and B. cinerea (Liu et al., 2019). These findings indicate that the Sscnd1 gene-silenced strains exhibit a defect in the compound appressorium formation possibly due to a defect in cell integrity, which caused a high sensitivity to environmental stressors. A mutant with a disruption in the secretory protein ScCa1 failed to form compound appressoria and was severely inhibited by osmotic stress conditions (Xiao et al., 2014). Similar to ScCa1, Sscnd1 was predicted to contain a signal peptide and an extracellular and non-membrane location. Additional studies are needed to explore whether the molecular role of Sscnd1 in compound appressorium formation is the same as that of ScCa1.

HIGS is a RNAi technology where small RNAs produced in plants can specifically silence the pathogen genes. It has been suggested to be an efficient tool for the potential control of various fungi in crops (Chen et al., 2016; Zhu et al., 2017; Qi et al., 2018, 2019; Xu et al., 2018). Excavation and functional
analysis of the virulence factors in *S. sclerotiorum*, such as OA (Cessna et al., 2006; Liang et al., 2015a,b), cell wall-degrading enzymes (CWDEs) (Yajima et al., 2009; Yu et al., 2016), secretory proteins (Guyon et al., 2014; Yang et al., 2018) and ROS (Kim et al., 2011; Xu and Chen, 2013), supply key HIGS targets for enhancing Sclerotinia resistance. Andrade et al. (2015) first proved that the HIGS-mediated chitin synthase gene (CHS) in tobacco enhanced resistance to *S. sclerotiorum* in the T1 generation. McCaghey et al. (2021) provided evidence supporting that *S. sclerotiorum* can uptake environmental RNAs and RNAi of oxaloacetate acetylhydrolase (Soah1) using HIGS reduced the pathogen aggressiveness. In this study, we selected Sscnd1 as the target gene to apply HIGS in *A. thaliana*. We transiently expressed the RNAi construct of Sscnd1 in *N. benthamiana* and stably expressed it in *A. thaliana*. The transgenic lines showed significantly enhanced resistance to *S. sclerotiorum*. The limitation of RNAi technologies is the potential off-target effect (Lundgren and Duan, 2013). Although there were four other homologous genes in *S. sclerotiorum*, Sscnd1 was sequence specific among its homologs in *A. thaliana* at the nucleotide level. Meanwhile, the sequence of Sscnd1 revealed relatively low similarity with *A. thaliana* genome. This sequence-specific prevents silencing of other homologous non-target genes by the application of RNAi (Nakayashiki and Nguyen, 2008). The random insertion rather than site-specific insertion of RNAi construct may interfere with the expression of related genes (Jia et al., 2017). However, different Sscnd1 RNAi strains and HIGS-Sscnd1 lines showed similar phenotypes for the pathogen virulence and compound appressorium formation, which makes the role of Sscnd1 persuasive.

Compound appressorium are hyphal tip-differentiated multicellular infection structures formed by many plant-pathogenic fungi on the host surface and is critical for penetrating into the host cells (Boenisch and Schäfer, 2011). Hu et al. (2020) proved that genes involved in urediniospore germination or appressorium formation can be used to manage Asian soybean rust (ASR) through HIGS in soybean. Mahto et al. (2020) found that silencing the CgCOM1 in chili and tomato suppressed the appressorium formation and mycelial growth of *Colletotrichum gloeosporioides*, resulting in reduced infection of plant tissues. The infection stages of *C. gloeosporioides* switches from biotrophic (conidia germination, formation of appressoria, penetration peg and primary hyphae) and necrotrophic (formation of secondary hyphae) phases (O’Connell et al., 2012). Similar to *C. gloeosporioides*, there may be a transition from a biotrophic to necrotrophic lifestyle in *S. sclerotiorum* (Kabbage et al., 2015; Liang and Rollins, 2018). Sscnd1 encoding a appressorium- related protein and function in the fungal full virulence in *S. sclerotiorum*. Silencing Sscnd1 in *A. thaliana* limited the compound appressorium formation during infection, and enhanced the host resistance. Meanwhile, the expression of several defense-related marker genes (AtPR1, AtPR2, AtPR5, and AtPDF1.2) showed no significant difference in HIGS-Sscnd1 lines from that in control lines, indicating that the reduced pathogenicity in HIGS-Sscnd1 lines is indeed caused by silencing of Sscnd1. These results prove the role of Sscnd1 in conferring resistance to *S. sclerotiorum* to host plants.

Many studies evident that target ds/siRNAs were presented in HIGS lines (Dou et al., 2020; Hu et al., 2020; Mahto et al., 2020; Singh et al., 2020; McCaghey et al., 2021), indicating that the uptake of ds/siRNA is likely a common occurrence in the fungal kingdom. It was shown that host *Arabidopsis* cells secrete exosome-like extracellular vesicles to deliver sRNAs into *B. cinerea*, and these sRNA-containing vesicles accumulate at the infection sites and are taken up by fungal cells, resulting in the silencing of fungal pathogenicity genes (Cai et al., 2018). Koch et al. (2020) also found that HIGS involves the transfer of dsRNA-derived siRNA via extracellular vesicles in *Arabidopsis*. Several studies suggest that host-derived siRNA is thought to translocate into pathogens via haustoria or similar structures, and silencing the highly expressed haustoria genes have been proved be more effective in HIGS application (Nowara et al., 2010; Yin et al., 2011; Panwar et al., 2013). However, the mechanism of host-derived RNA translocation across the plant cells to *S. sclerotiorum* cells is yet to be determined.

In conclusion, we found that Sscnd1 is required for compound appressorium formation, cell integrity and fungal full virulence in *S. sclerotiorum* and is a potential target for improving Sclerotinia resistance in crops via HIGS.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

WQ planned and designed the research. YD, YC, BY, HL, and MD performed research. XM conducted RNA-seq analyses. WQ, HW, and YD analyzed and validated the data. YD and WQ wrote the original draft of the manuscript. All authors discussed the data, edited, and approved the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.693334/full#supplementary-material
Supplementary Figure 1 | Expression of MAS-related genes in S. sclerotiorum during infection. Expression of five MAS-related genes in S. sclerotiorum during the inoculation of Brassica oleracea leaves (A) and stems (B), as revealed by RNA-seq in our previous study (Mei et al., 2016; Ding et al., 2019). (C) Top 10 expressed S. sclerotiorum genes during the inoculation of B. oleracea leaves. RL, resistant B. oleracea leaf; SL, susceptible B. oleracea leaf; RIS, resistant B. oleracea stem; RS, susceptible B. oleracea stem; 0 h, pre-inoculation; 6 h, 12 h post-inoculation; 12 h, 12 h post-inoculation; 24 h, 24 h post-inoculation.

Supplementary Figure 2 | Expression analysis of Sscnd1 and its homologous genes during the inoculation of rapeseed leaves. The quantity of Sstub1 was used to normalize the expression levels of every gene in different samples. The relative expression of S21G_11468 at 0 h was set as one. Mean values from three independent assays were used to construct the heatmap.

Supplementary Figure 3 | Expression analysis of the Sscnd1 homologous genes in Sscnd1 gene-silenced strains. (A) Multiple alignment of the amplified 418 bp of Sscnd1 with the other four homologous genes in S. sclerotiorum. (B) Relative transcript levels of the four homologous genes in the Sscnd1 gene-silenced strains. The quantity of Sstub1 was used to normalize the expression levels of Sscnd1 in different samples. Error bars indicate the standard deviation of three independent assays. Differences in every gene group were assessed using Tukey’s HSD test, respectively. Different letters indicate statistical significance at the 0.05 level ($P < 0.05$).

Supplementary Figure 4 | The correlation between the Sscnd1 expression and the compound appressorium numbers. Sscnd1-9, Sscnd1-10, Sscnd1-15, Sscnd1-16, Sscnd1-17, and Sscnd1-20 were six Sscnd1 gene-silenced strains.

Supplementary Figure 5 | Expression changes of Sscnd1 and its homologous genes in Sscnd1-9 and Sscnd1-20, as revealed by RNA-seq. FC: Fold change (Sscnd1-9 or Sscnd1-20 vs. wild-type strain).

Supplementary Figure 6 | Expression changes as estimated by RNA-Seq (black bars) and qRT-PCR (gray bars) for 15 S. sclerotiorum genes during the inoculation of leaves 48 hpi with B. oleracea post-inoculation; 12 h, 12 h post-inoculation; 24 h, 24 h post-inoculation.

Supplementary Figure 7 | Functional assessment of Sscnd1 in S. sclerotiorum pathogenicity determined by TRV-mediated HIGS. (A) Disease phenotypes of Mock (mock-inoculated with FES buffer)-, TRV::GFP-, TRV: Sscnd1-1- and TRV: Sscnd1-2-inoculated N. benthamiana leaves at 48 hpi with S. sclerotiorum strain 1980. (B) Quantification of the lesion area in (A). Error bars indicate the standard deviation for five replicates. Differences were assessed using Tukey’s HSD test. Different letters indicate statistical significance at the 0.05 level ($P < 0.05$). (C) Relative transcript levels of Sscnd1 in the Mock-, TRV::GFP-, TRV::Sscnd1-1- and TRV::Sscnd1-2-inoculated N. benthamiana leaves 48 hpi with S. sclerotiorum strain 1980. The quantity of Sstub1 was used to normalize the expression levels of Sscnd1 in different samples. Error bars indicate the standard deviation of three independent assays. Differences were assessed using Tukey’s HSD test. Different letters indicate statistical significance at the 0.05 level ($P < 0.05$).

Supplementary Figure 8 | Morphological characterizations of transgenic and control A. thaliana lines.

Supplementary Figure 9 | The expression of specific siRNA of Sscnd1 gene (Sscnd1-siRNA) in HIGS-Sscnd1 transgenic A. thaliana lines as determined by stem-loop qRT-PCR analysis. The cDNA harboring the loop sequence was used for stem-loop qRT-PCR analysis in Col-0 (wild-type A. thaliana), EV (A. thaliana lines containing the empty vector) and HIGS-Sscnd1 A. thaliana lines. The quantity of AtU6-26 was used to normalize the expression levels of Sscnd1 siRNA in different samples. Error bars indicate the standard deviation of three independent assays.

Supplementary Figure 10 | Gene expression analysis. (A) Relative transcript levels of AtPR2, AtPDF1.2, AtPDF1.3, and AtPDF1.2 genes in the Col-0 (wild-type A. thaliana), EV (A. thaliana lines containing the empty vector) and HIGS-Sscnd1 transgenic A. thaliana lines. The quantity of AtActin8 was used to normalize the expression levels of every gene in different samples. Error bars indicate the standard deviation of three independent assays. The relative expression of every gene in EV was set as one. (B) Relative transcript levels of Sscnd1 homologous genes in the S. sclerotiorum-infected HIGS-Sscnd1 lines at 24 hpi. The quantity of Sstub1 was used to normalize the expression levels of every gene in different samples. Error bars indicate the standard deviation of three independent assays. The relative expression of every gene in EV was set as one. Differences in every gene group were assessed using Tukey’s HSD test, respectively. Different letters indicate statistical significance at the 0.05 level ($P < 0.05$).

Supplementary Figure 11 | Expression of the HIGS-Sscnd1 construct in A. thaliana Col-0 enhances resistance against Botrytis cinerea. (A) Disease phenotypes of Col-0 (wild-type A. thaliana), EV (empty vector line) and HIGS transgenic lines (HIGS-Sscnd1-25, HIGS-Sscnd1-39, and HIGS-Sscnd1-42) after inoculation with B. cinerea strain B5010. Photographs were taken at 24 hpi. (B) Quantification of the lesion area in (A). Error bars indicate the standard deviation for five replicates. Differences were assessed using Tukey’s HSD test. Different letters indicate statistical significance at the 0.05 level ($P < 0.05$). (C) Relative transcript levels of BC1G_08931 in the leaves of Col-0, EV, and HIGS transgenic lines 24 hpi with B. cinerea strain B5010. Total BC1G_08931 cDNA abundance in the samples was normalized using BcActin gene as a control. Error bars indicate the standard deviation of three independent assays. Differences were assessed using Tukey’s HSD test. Different letters indicate statistical significance at the 0.05 level ($P < 0.05$).

Supplementary Table 1 | Information on the primers used in this study.

Supplementary Table 2 | Information of the differentially expressed genes (DEGs) (Sscnd1-19 and Sscnd1-20 vs. WT).

Supplementary Table 3 | Segregation analyses and lesion areas of Col-0 (wild-type A. thaliana), EV (empty vector line) and HIGS transgenic lines (HIGS-Sscnd1-25, HIGS-Sscnd1-39, and HIGS-Sscnd1-42) after inoculation with S. sclerotiorum.

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