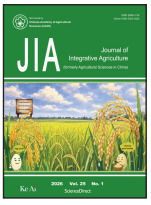
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RESEARCH ARTICLE

SsBMR1 as a putative ABC transporter is required for pathogenesis by promoting antioxidant export and antifungal resistance in *Sclerotinia sclerotiorum*

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Highlights

- Silencing the *SsBMR1* gene in *Sclerotinia sclerotiorum* significantly reduces fungal growth, infection, sclerotia formation, and virulence.
- Host-induced gene silencing (HIGS) of *SsBMR1* enhances plant resistance, providing a potential target for managing Sclerotinia stem rot (SSR) disease in dicotyledons.
- *SsBMR1* is involved in antioxidant and toxin transport, influencing fungal defense mechanisms and resistance to plant antifungal substances and fungicides.

Abstract

The plant pathogenic fungus *Sclerotinia sclerotiorum* is the causative agent of Sclerotinia stem rot (SSR) disease in most dicotyledons. Among the various proteins involved in drug efflux or substance transport, ATP-binding cassette (ABC) transporters constitute a superfamily of membrane-bound proteins that may play a crucial role in the survival of *S. sclerotiorum*. However, the expression patterns and functions of ABC transporter genes in *S. sclerotiorum* remain largely uncharacterized. This study characterized a highly expressed *S. sclerotiorum* ABC transporter gene during inoculation on host plants, *SsBMR1*. Silencing *SsBMR1* resulted in a significant reduction in hyphal growth, infection cushion development, sclerotia formation, and virulence. Moreover, host-induced gene silencing (HIGS) of *SsBMR1* significantly enhanced plant resistance. Transcriptome and metabolomics analyses suggested that *SsBMR1* is involved in antioxidant and toxin transport, thereby influencing fungal defense and cell rescue mechanisms. In comparison to the wild-type strain, *SsBMR1* gene-silenced transformants exhibited a diminished response to extracellar oxidative stress and a decreased exporting of antioxidant glutathione. Tolerance assays further demonstrated the crucial role of *SsBMR1* in conferring resistance to the plant antifungal substances, camalexin and brassinin, as well as certain fungicides. Furthermore, *SsBMR1* gene-silenced transformants showed enhanced repression on virulence when sprayed with camalexin and brassinin on the leaves. Thus, *SsBMR1* likely contributes to virulence by facilitating the export of antioxidant and providing resistance against antifungal agents. The findings of this study provide valuable insights that could contribute to the development of novel management techniques for SSR.

Keywords: ABC transporter, antifungal resistance, glutathione, pathogenesis, *Sclerotinia sclerotiorum*

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1. Introduction

Sclerotinia sclerotiorum is a fungal pathogen notorious for inducing a spectrum of symptoms in plants such as water-soaked lesions, wilting, rot, soft rot, and shriveling, frequently culminating in plant death (Jahan *et al.* 2022). This widespread fungus poses a significant threat to agricultural production. *Sclerotinia sclerotiorum* stands out among plant pathogens due to its ability to infect a broad range of plants, including 425 species across 74 different families, such as sunflowers, soybeans, canola, and various dicotyledons (Derbyshire *et al.* 2022; Hossain *et al.* 2023). Recent research has also identified *S. sclerotiorum* as an endophytic pathogen of some monocotyledonous plants, such as rice, wheat, maize, barley, and oats (Tian *et al.* 2020). Although *S. sclerotiorum* has long been recognized as a typical necrotrophic pathogen, recent studies have revealed a biotrophic phase during the initial infection stage of the host (Kabbage *et al.* 2015; Liang and Rollins 2018). Within this phase, *S. sclerotiorum* tactfully mitigates the host's physical and chemical defense mechanisms while up-regulating effector genes to disrupt host signal transduction, confound host recognition, and dampen host defense responses (Liang and Rollins 2018; Ding *et al.* 2021). Following successful colonization, *S. sclerotiorum* transitions to the necrotrophic phase, inciting a marked increase in host reactive oxygen species (ROS) levels, and releasing a plethora of lytic enzymes that compromise the structural integrity of the subcuticular epidermal walls, thereby initiating apoptotic cell death (Williams *et al.* 2011; Kabbage *et al.* 2013). The multifaceted pathogenic strategy employed by *S. sclerotiorum* amplifies the challenges associated with its prevention and control.

Currently, the primary strategies for managing *S. sclerotiorum* include cultivation management, the use of chemical and biological controls, and the development of disease-resistant plant varieties. However, the effectiveness of cultivation management is restricted due to the extensive host range of *S. sclerotiorum* and the high survivability of its sclerotia in various environmental conditions. Furthermore, a dearth of effective host resistance further hampers the exploration and application of disease-resistant genes, thereby stifling advancements in crop breeding research (Mei *et al.* 2020). The deployment of chemical and biological control methods often results in subpar effects owing to inaccurate timing and incorrect application practices. Additionally, the use of fungicides escalates production costs and exacerbates environmental pollution (Partridge *et al.* 2006). Additionally, certain ABC transporters, also known as ATP-binding cassette transporters (ABCT), enable fungi to develop multidrug resistances by actively effluxing fungicides, thus diminishing the efficacy of fungicide application (Moreno *et al.* 2019).

ABC transporter represents one of the most widely distributed protein families in living organisms. These proteins are characterized by highly conserved nucleotide-binding domains (NBDs) and transmembrane binding domains (TMDs) (El-Awady *et al.* 2017; Víglaš and Olejníková 2021). ABC transporters are driven by ATP hydrolysis to transport various substrates across membranes (Prasad and Goffeau 2012; Baral 2017). Fungal ABC transporters are categorized into eight subfamilies: ABCA, ABCB/MDR (multidrug resistance), ABCC/MRP (multidrug resistance associated proteins), ABCD/ALD (adrenoleukodystrophy), ABCE, ABCF, ABCG/PDR (pleiotropic drug resistance), and ABCI (Víglaš and Olejníková 2021). Most of the researches conducted on fungal ABC proteins focus on their function in terms of antifungal efflux (Fachin *et al.* 2006; Song *et al.* 2013). In *S. cerevisiae*, ABCE and ABCF proteins are regarded necessary for cell viability (Kovalchuk and Driessen 2010). ABCG is one of the largest subfamilies, which can serve as both importers and exporters (effux pumps), capable of transporting various xenobiotics such as anti-fungal drugs, fungicides, herbicides, antibiotics, and phytoalexins (Prasad and Goffeau 2012). In addition, a PDR-type ABC transporter BcatrB, in *B. cinerea*, has identified as a virulence factor in *A. thaliana* and grapevine (Schoonbeek *et al.* 2001, 2003; Stefanato *et al.* 2009). MgAtr4 in *M. graminicola* was found involved in the fungal colonization of substomatal cavities in wheat leaves (Stergiopoulos *et al.* 2003). Despite the identification of potential ABC transporter genes in the *S. sclerotiorum* genome (Amselem *et al.* 2011), their expression profiles and functional roles remain to be elucidated.

In this study, we conducted an extensive investigation of the gene structure and expression profile of ABC transporters in *S. sclerotiorum* during the infection process on different host plants. Among these transporters, we placed particular emphasis on a highly expressed ABC gene, namely *SsBMR1*, for comprehensive gene function analysis. Our research reveals that *SsBMR1* plays a significant role in the virulence of *S. sclerotiorum* by facilitating the efflux of antioxidants and conferring resistance against antifungal agents. These findings provide valuable insights into the involvement of ABC transporters as virulence factors in the pathogenic mechanism of *S. sclerotiorum*, offering potential avenues for researchers to develop effective strategies to combat plant diseases induced by this formidable pathogen.

2. Materials and methods

2.1. Fungal strains, plants, and culture conditions

The *S. sclerotiorum* wild-type 1980 was cultured on potato dextrose agar (PDA) plates (Difco Laboratories, Detroit) (Godoy *et al.* 1990). *SsBMR1* gene-silenced transformants were cultured on PDA plates supplemented with hygromycin B antibiotic at a final concentration of 100 μg mL^{-1} (Calbiochem, San Diego, CA). *Arabidopsis thaliana* wild-type Col-0 (Columbia zero background ecotype) and transgenic lines were grown under controlled environment conditions with 16-h/23°C days and 8-h/16°C nights, and a light intensity of 100 μmol m^{-2} s^{-1}. *Brassica napus* cv. Zhongshuang 11 (rapeseed) plants were cultivated in the field.

2.2. Identification and structural analysis of ABC transporter proteins in *S. sclerotiorum*

The genome sequence of *S. sclerotiorum* was downloaded from the database (http://fungidb.org/common/downloads/

Current_Release/Ssclerotiorum_/). The conserved ABC transporter domain (PF00005) was used to identify ABC transporter proteins in *S. sclerotiorum* using the HMMER (V3.3.2) software (Finn *et al.* 2011) (the e-value was set to 1×10^{-5}) and Pfam (http://pfam.xfam.org/) (Finn *et al.* 2016) databases. The accuracy of these identified proteins was further confirmed using the SMART (Letunic *et al.* 2012) and INTERPROSCAN (Jones *et al.* 2014) programs. A phylogenetic tree was constructed using the neighbour-joining method in MEGA 7.0 (http://www.megasoftware.net/), and the bootstrap test was replicated 1,000 times. PROTTER (http://wlab.ethz.ch/protter/# (accessed on 14 November 2022)) was used to predict protein sequence features.

2.3. Plasmid construction and transformation

The *SsBMR1* gene-silencing vector was constructed based on the plasmid pCIT (Yu *et al.* 2017). Fragments of both the sense and antisense strands of *SsBMR1*, spanning a length of 376 bp, were inserted into the respective clone sites of pCIT. Subsequently, the hygromycin resistance gene cassette from pSKH (Hamid *et al.* 2013) was incorporated into the vector. The resulting construct, pSibmr1, was then transformed into the wild-type *S. sclerotiorum* 1980 using the polyethylene glycol (PEG)-mediated transformation protocol (Rollins 2003).

For the generation of VIGS-*SsBMR1*, the 376 bp RNAi fragment was inserted into the TRV2 vector (Liu and Page 2008) to create the VIGS constructs TRV2::*SsBMR1* along with TRV2 empty vector as a control. The infiltration of *Nictiana benthamiana* plants was performed following the method described by Liu and Page (2008).

To generate host-induced gene silencing (HIGS)-*SsBMR1*, the sense and antisense fragments of *SsBMR1* were flanked by the intron 3 of the malate synthase gene from *A. thaliana* (i3). The resulting '*SsBMR1*+-intron-*SsBMR1*-gene' cassette was then cloned into a plasmid containing the red fluorescent protein (DS Red), namely pBinGlyRed3. The HIGS construct HIGS-*SsBMR1* was introduced into the *Agrobacterium tumefaciens* strain GV3101 through electroporation (Wise *et al.* 2006), and subsequently transformed into *A. thaliana* Col-0 using the floral dip-mediated transformation protocol (Clough and Bent 1998).

2.4. Analysis of the expression of *SsBMR1* during different developmental and infection stages of *S. sclerotiorum*

The *S. sclerotiorum* mycelia were harvested during different developmental stages: hyphae stage (2 days post-inoculation, dpi), initial sclerotial stage (3 dpi), developed sclerotial stage (5 dpi), and mature sclerotial stage (8 dpi), following the method described by Yu *et al.* (2017). To evaluate the expression of *SsBMR1* during the infection process *B. napus* cv. Zhongshuang 11 (rapeseed) leaves that were inoculated and hyphae growing on MM (minimal medium) plates were collected at 0, 12, 24, 36, and 48 h post-inoculation (hpi). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and then first-strand cDNA was synthesized for quantitative real-time reverse transcription-PCR (qRT-PCR). The qRT-PCR was performed using the Bio-Rad CFX96 Real-Time System (USA) and Quantitect SYBR Green PCR master mix (Bio-Rad, USA) according to the manufacturer's instructions. The β-tubulin gene *Sstub1* (SS1G_04652) was utilized as the internal reference for normalization. The transcript levels of the gene were calculated 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 primer sequences are listed in Appendix A.

2.5. Phenotypic characterization of transformants

To assess the difference in the growth rate of the wild-type 1980 and *SsBMR1* gene-silenced transformants, the strains were cultured on PDA plates for 2 days. The diameter was measured every 12 h until the mycelium reached the edges of the plates. The strains were then cultivated for an additional 25 days, and the number and weight of mature sclerotia were analyzed. To compare the morphology of hyphal tips, the wild-type 1980 and *SsBMR1* gene-silenced transformants were cultured on PDA for 24 h at 22°C, and the morphology of the hyphal tips was observed using a light microscope.

The 0.6-cm agar plugs from the actively growing colony edges of *SsBMR1* gene-silenced transformants were used to inoculate *B. napus* leaves and stems (*in vitro*). In addition, 0.2-cm agar plugs colonized by mycelium were inoculated into *A. thaliana* Col-0 seedlings (*in vivo*). The inoculated *B. napus* leaves, stems, and *A. thaliana* Col-0 plants were maintained at 22°C and 90% relative humidity (RH). The lesion area or lesion length was measured at 2 dpi for *B. napus* leaves, 3 dpi for *B. napus* stems, and 1 dpi for *A. thaliana* plants, respectively. Each transformant was assessed with at least three leaves/replication, and the experiments were conducted five times. The *S. sclerotiorum* wild-type 1980 was used as the control. The lesion area (S, cm^2) was calculated using the formula $S=\pi ab/4$, where *a* and *b* represent the long and short diameters of an approximately elliptical lesion.

To evaluate the oxidative stress tolerance in the *S. sclerotiorum* wild-type 1980 and *SsBMR1* gene-silenced transformants, the strains were cultured on PDA plates supplemented with 4, 8, and 12 mmol L^{-1} hydrogen peroxide (H_2O_2), respectively. The inhibition of hyphal growth was calculated by measuring the growth rate every 12 h until the mycelium reached the margins of the plates. The wild-type 1980 and *SsBMR1* gene-silenced transformants were further inoculated on PDA containing the plant antifungal substances 0.5 mg mL^{-1} camalexin (SML1016, Sigma), 0.5 mg mL^{-1} brassinin (SML1635, Sigma), certain fungicides, 1 μg mL^{-1} triazolone, 0.5 μg mL^{-1} carbendazim, 1 μg mL^{-1} boscalid, and 0.5 μg mL^{-1} azoxystrobin individually. The growth inhibition rate was calculated. Furthermore, wild-type 1980 and *SsBMR1* gene-silenced transformants were inoculated on the leaves pre-incubated with 0.5 mg mL^{-1} camalexin and 0.5 mg mL^{-1} brassinin for 30 min, the lesion area (S, cm^2) was calculated at 48 hpi. The experiment was repeated for five times.

The strains were inoculated onto parafilm-covered PDA plates and *A. thaliana* leaves to assess the formation of compound appressoria. At 8 hpi, the plugs were removed.

The parafilm was stained with 5% trypan blue and then washed with distilled water for three times. The inoculated *A. thaliana* leaves were stained with 5% trypan for 12 h and then cleared with ethanol/acetic acid (3:1, v/v) solution for 12 h. The compound appressoria were observed using a microscope. The experiment was repeated three times independently.

2.6. Resistance assay of transgenic plants

The VIGS-*SsBMR1* transgenic leaves of *N. benthamiana*, and the HIGS-*SsBMR1* transgenic leaves of *A. thaliana* were inoculated with the *S. sclerotiorum* wild-type 1980. The lesion area was recorded after 48 hpi for *N. benthamiana* and 24 hpi in case of *A. thaliana*. The experiments were performed for three times with at least three leaves/each line in one replicate.

2.7. Transcriptome analysis

Four-week-old lines of *A. thaliana*, including the wild-type Col-0 and the HIGS-*SsBMR1* transgenic line L16, were inoculated with the *S. sclerotiorum* wild-type 1980 and the *SsBMR1* gene-silenced tranformant Sibmr1-1, respectively. The inoculated tissues were collected at 12 hpi. Library construction and Illumina sequencing were carried out by BMK (Qingdao, China). The sequencing reads were aligned to the genome of *S. sclerotiorum* 1980 (http://fungidb.org/common/downloads/Current_Release/Ssclerotiorum_/), using Hisat2 (Kim *et al.* 2015), and only uniquely mapped reads with up to two mismatches were retained. Read counts for each gene were then calculated using FeatureCounts (Liao *et al.* 2014), and the gene expression levels were normalized into FPKM (fragments per kilobase of transcript per million) using the R package limma (Ritchie *et al.* 2015). DEGs (differentially expressed genes) were identified using DESeq2 (Love *et al.* 2014) with default settings, considering genes with at least a two-fold change in expression and a q-value less than 0.05 (FDR, corrected from *P*-values by the Benjamini-Hochberg testing) as significant. Furthermore, significantly enriched FunCat categories of DEGs were determined using FungiFun (https://sbi.hki-jena.de/fungifun/fungifun.php) (Priebe *et al.* 2015), with a significance threshold of $P \leq 0.05$.

2.8. Metabolomics analysis

Metabolome analysis of the mycelia of *S. sclerotiorum* wild-type 1980 and the *SsBMR1* gene-silenced tranformant Sibmr1-1 was conducted at 2, 7, and 15 dpi on PDA plates. Three biological replicates were performed for each time point. The fresh mycelia were freeze-dried, weighed, and crushed into powder. The powder was then homogenized in an aqueous solution of formic acid (0.1%, v/v) in aqueous methanol (1 mL; 80%, v/v) and subjected to sonication (KQ-100E, Kunshan, China) for 1 h. After centrifugation at 10,000 r min^{-1} for 15 min, the supernatants were filtered using a 0.22-μm nylon filter and analyzed using ultra-high-performance liquid chromatography–heated electrospray ionization–tandem mass spectrometry (UPLC–HESI–MS/MS). Metabolite analysis was performed using a Dionex UltiMateTM 3000 UHPLC System (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Thermo Scientific Q-Exactive System equipped with an S-Lens ionizer source (Thermo Scientific, USA) in a negative mode, following the method described by Shen *et al.* (2021). The metabolites were analyzed using MS-DIAL version 4.18 software with a MassBank negative database (Tsugawa *et al.* 2019) and further confirmed according to Yin *et al.* (2019). Differentially accumulated metabolites (DAMs) were identified based on the variable importance of the projection (variable important in projection, VIP) value≥1 and $|\log_2(\text{fold change})| \geq 1$.

2.9. Measurement of glutathione and H_2O_2 content

Cellular and released glutathione contents were determined using the glutathione assay kit (A006-2-1; Nanjing Jiancheng Bioengineering Institute) following the manufacturer's protocol. The wild-type 1980 and *SsBMR1* gene-silenced transformants were cultured in potato dextrose broth (PDB) medium containing 4 mmol L^{-1} H_2O_2 for 24 h, and then both mycelium and the culture medium were collected for GSH content analysis. Quantification of H_2O_2 content in the mycelium was performed using the Hydrogen Peroxide Assay Kit (A064-1-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. The experiment was repeated three times for each strain.

3. Results

3.1. Identification and characteristics of ABC transporter gene *SsBMR1* in *S. sclerotiorum*

ABC transporters play crucial roles in the complex process of controlling fungal development and pathogenicity (Víglaš and Olejníková 2021). A total of 33 ABC transporter genes with the ABC transporter domain were identified in *S. sclerotiorum* through HMMsearch by using PF00005 as the query and subsequent domain verification. These genes were further classified into five subfamilies: ABCB/MDR (14 members), ABCG/PDR (pleiotropic drug resistance) (10 members), ABCC/MRP (6 members), ABCD (2 members), and ABCA (1 member) (Fig. 1-A; Appendix B). To gain insights into the functions of these ABC transporter genes in *S. sclerotiorum*, we analyzed their expression patterns by collecting gene expression data during inoculation on three host plants (*B. napus*, *Brassica oleracea*, and *A. thaliana*) using RNA-seq. Most of the 33 ABC transporter genes exhibited increased expression levels during inoculation compared to that growing on PDA medium. Notably, SS1G_04483 showed the most significant increase in expression across all three different host plants during inoculation (Fig. 1-A). Sequence analysis revealed that SS1G_04483 has an open reading frame (ORF) of 4,155 bp, encoding a putative protein composed of 1384 amino acids with a molecular weight of 154.3 kD and an isoelectric point (pI) of 8.30 (Appendix B). The prediction of transmembrane regions indicated that SS1G_04483 encodes a protein with 13 transmembrane domains (Fig. 1-B). We constructed the phylogenetic tree with 31 genes from other fungi species. Phylogenetic analyses showed that SS1G_04483 was closely related to *Botryotinia fuckeliana* BMR1 (Appendix C). Consequently,

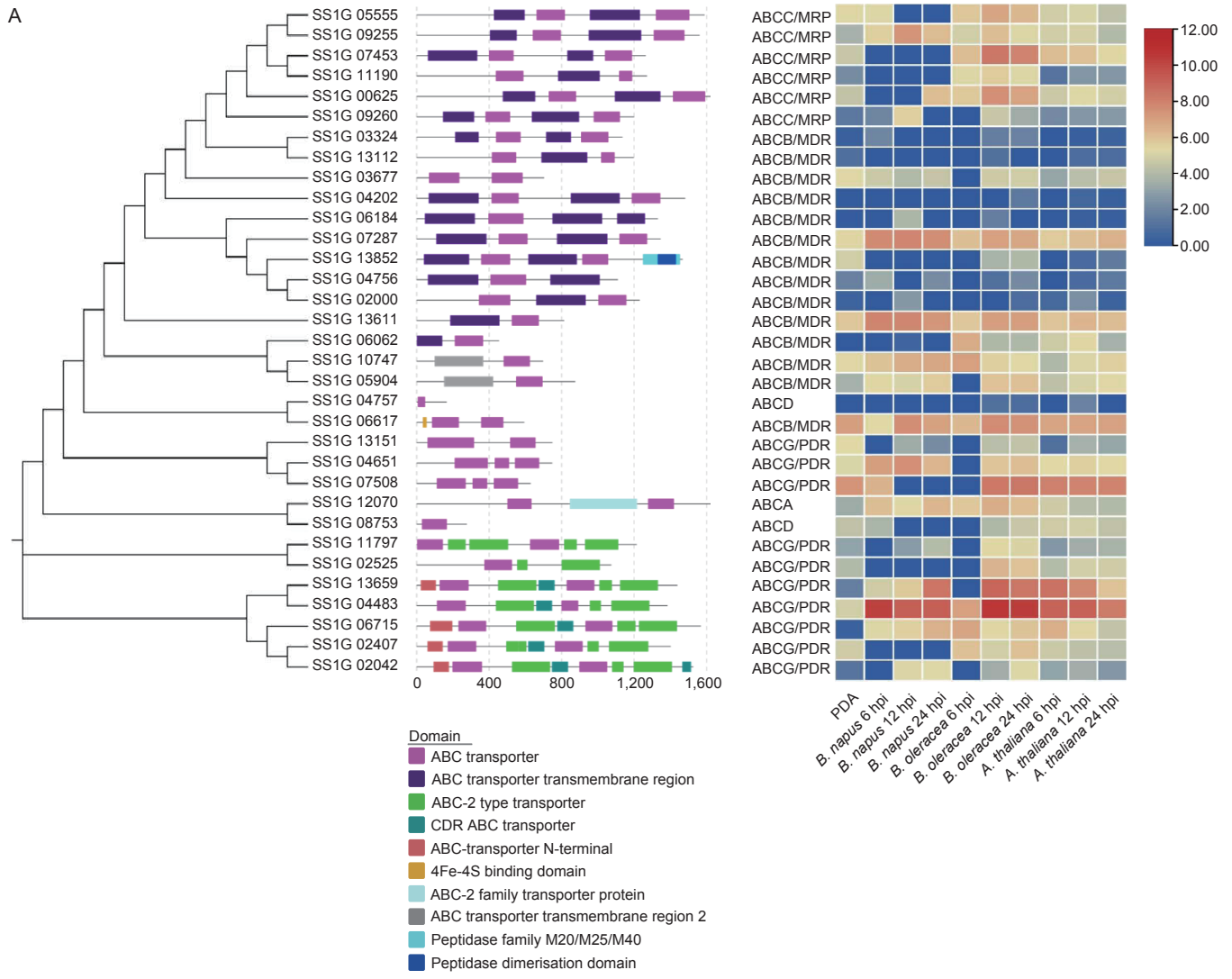


Fig. 1 Identification and expression analysis of ABC transporter (ABCT) genes in *Sclerotinia sclerotiorum*. A, conserved domain structures of the identified SsABCTs and their expression profiles are depicted in the heat map. Different motifs are represented by boxes of varying colors. PDA, hyphal grow on PDA for two days; *B. napus*, *Brassica napus*; *B. oleracea*, *Brassica oleracea*; *A. thaliana*, *Arabidopsis thaliana*; hpi, hours post inoculation. B, predicted membrane topology of SS1G_04483 as depicted using the PROTTER webserver. The transmembrane domains are based on UniProt features and are numbered from 1 to 13.

we designated SS1G_04483 as *SsBMR1*.

To investigate the transcript levels of *SsBMR1* at different stages of *S. sclerotiorum* development and infection, qRT-PCR was performed. It revealed a gradual induction of *SsBMR1* transcript levels during the sclerotial development stage (Fig. 2-A). Furthermore, during the inoculation on *B. napus*, *SsBMR1* exhibited early induction at 12 hpi, reaching its maximum level of up to 4.6-fold at 36 hpi compared to growth on the MM medium (Fig. 2-B). These results suggest that the transcription of *SsBMR1* is induced during both sclerotia development and infection stages.

3.2. *SsBMR1* plays crucial roles in hyphal development, infection cushion and sclerotia formation

The *SsBMR1* gene-silencing vector, pSibmr1, was constructed and transformed into wild-type *S. sclerotiorum* 1980. Two silenced transformants Sibmr1-1 and Sibmr1-2 were generated, and the expression of *SsBMR1* in these silenced transformants was confirmed using qRT-PCR with gene-specific primers. The gene silencing elicited a substantial decrease in *SsBMR1* transcript levels in Sibmr1-1 and Sibmr1-2, which were reduced to 21 and 28% of the levels detected in the wild-type strain 1980, respectively (Fig. 2-C). To analyze the effects of *SsBMR1* on fungal growth and

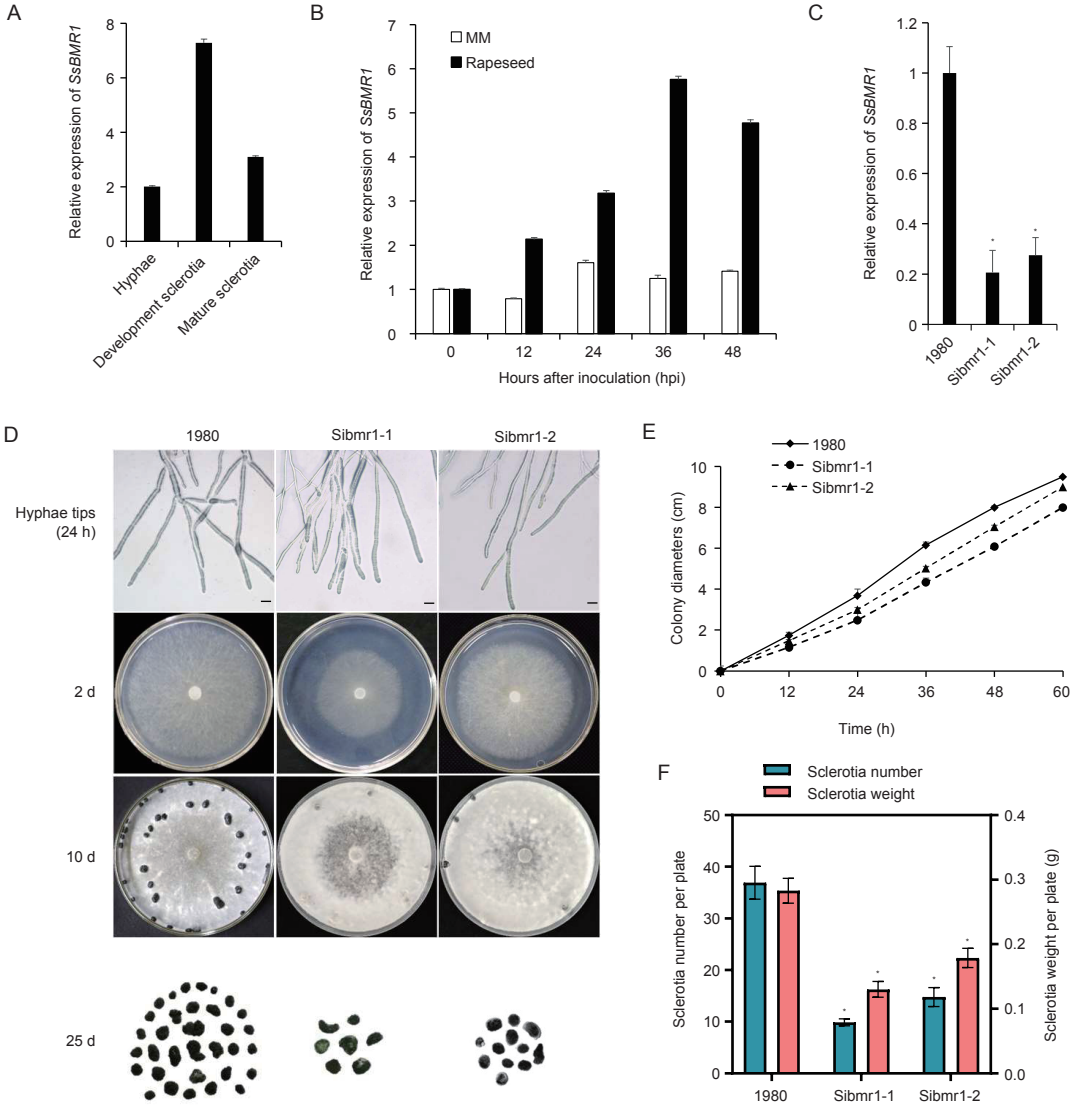


Fig. 2 Effects of *SsBMR1* silencing on hyphal development and sclerotia formation in *S. sclerotiorum*. A, relative expression level of *SsBMR1* gene at different hyphae growth stages. The expression levels were normalized using the *SsTub1* gene as an internal control. B, relative expression of *SsBMR1* gene at different time points after inoculation and growing on the MM (minimal medium). The expression levels were normalized using the *SsTub1* gene as an internal control. The relative expression of *SsBMR1* at 0 h was set as one. C, relative expression level of *SsBMR1* in gene-silenced strains. The expression levels were normalized using the *SsTub1* gene as an internal control. D, phenotypes of the mycelium and sclerotia in the *SsBMR1* gene-silenced transformants and the wild-type 1980. One representative biological replicate is presented out of three replicates. E, radial growth of the *SsBMR1* gene-silenced transformants and 1980 on potato dextrose agar (PDA). F, number and weight of sclerotia in the *SsBMR1* gene-silenced strains and 1980 at day 25 are presented. The data represent the mean±SD (n=3 biological replicates). Asterisks (*) indicate a significant difference from 1980 at a P-value<0.05, determined using a two-tailed t-test.

sclerotia formation, the transformants Sibmr1-1 and Sibmr1-2, along with the wild-type 1980, were incubated on PDA plates. The hyphal tips of these different strains were observed under a microscope. There were no significant differences in the morphology and branching of mycelium between the *SsBMR1* gene-silenced transformants and the wild-type 1980, suggesting that the *SsBMR1* gene may not play a role in mycelial morphology in *S. sclerotiorum* (Fig. 2-D). However, the growth of hyphae was slower in the *SsBMR1* gene-silenced transformants compared to wild-type 1980 (Fig. 2-D and E). The colony diameter of Sibmr1-1 decreased by 27.2% on average, while Sibmr1-2 decreased by 13.8% on average (Fig. 2-E). To investigate the involvement of *SsBMR1* in infection cushion development, the wild-type 1980 and the *SsBMR1* gene-silenced transformants were inoculated on parafilm-overlaid PDA and *A. thaliana* leaves. It was observed that both Sibmr1-1 and Sibmr1-2 produced fewer compound appressoria compared to the wild-type 1980 (Appendix D). This indicates that *SsBMR1* is associated with compound appressorium formation in *S. sclerotiorum*.

To examine the role of *SsBMR1* in sclerotia formation, Sibmr1-1, Sibmr1-2, and the wild-type 1980 were cultured on PDA until 25 days. At 10 dpi, the wild-type 1980 exhibited a substantial number of mature sclerotia, whereas the formation of sclerotia was delayed in Sibmr1-1 and Sibmr1-2 (Fig. 2-D). The number and weight of sclerotia were determined at 25 dpi. Remarkably, both the number and mass of sclerotia formed by Sibmr1-1 and Sibmr1-2 were significantly reduced compared to the wild-type 1980 (Fig. 2-F). Specifically, Sibmr1-1 produced 14.6 with 0.16 g sclerotia per plate, and Sibmr1-2 produced 16.1 with 0.17 g sclerotia per plate. While, 1980 produced 36.9 with 0.29 g sclerotia per plate. These findings suggest that inhibiting the expression of *SsBMR1* in *S. sclerotiorum* impairs hyphal and sclerotia development.

3.3. *SsBMR1* is required for full virulence in *S. sclerotiorum*

To investigate the role of *SsBMR1* in the pathogenicity of *S. sclerotiorum*, leaves and stems of *B. napus* cv. Zhongshuang 11 as well as seedlings of *A. thaliana* Col-0, were inoculated with plugs of Sibmr1-1, Sibmr1-2, and the wild-type 1980. Comparison with 1980 revealed that the detached leaves and stems of rapeseed challenged with Sibmr1-1 and Sibmr1-2 exhibited smaller disease symptoms (Fig. 3-A and B). At 48 hpi, Sibmr1-1 and Sibmr1-2 caused average lesion areas of 8.31 and 8.54 cm^2, respectively, on rapeseed leaves. This represents a significant reduction of 39.0 and 37.2% compared to the average lesion area of 13.62 cm^2 caused by 1980. Similarly, the average lesion length on rapeseed stems induced by Sibmr1-1 and Sibmr1-2 was 3.09 cm and 3.48 cm, respectively, at 72 hpi. These results show a significant decrease of 50.6 and 44.4% compared to the average lesion length of 6.26 cm caused by 1980. To further assess the reduced pathogenicity of Sibmr1-1 and Sibmr1-2, we challenged seedlings of *A. thaliana* Col-0 with the strains *in vivo*. The disease symptoms caused by Sibmr1-1 and Sibmr1-2 were smaller than those caused by 1980 (Fig. 3-C). At 24 hpi, Sibmr1-1 and Sibmr1-2 induced average lesion areas of 0.48 and 0.51 cm^2, respectively. This represents a significant decrease of 48.8 and 45.6% compared to 1980, which had an average lesion area of 0.93 cm^2. These observations strongly suggest that *SsBMR1* is required for the pathogenicity of *S. sclerotiorum*, and the silencing of this gene significantly impairs the fungus's virulence.

3.4. Host induced *SsBMR1* silencing plants inhibits *S. sclerotiorum* disease

Using a TRV-based HIGS system, the functional role of *SsBMR1* in *S. sclerotiorum* virulence was further assessed. A random fragment of *SsBMR1* was selected to construct a VIGS vector for TRV-based HIGS analysis. Tobacco plants infiltrated with the TRV2::GFP vector (CK) displayed significant disease symptoms when inoculated with 1980. In contrast, plants infiltrated with VIGS-*SsBMR1* showed smaller disease symptoms (Appendix E). The lesion area on the leaves infiltrated with VIGS-*SsBMR1* was 5.43 cm^2 on average, and 7.20 cm^2 for the CK.

To confirm the stable inheritance of HIGS-*SsBMR1*-mediated resistance in higher generation transgenic plants, two stable transgenic lines of *A. thaliana* (L16 and L20) expressing the HIGS-*SsBMR1* vector from the T_3 generation were selected and challenged with the *S. sclerotiorum* wild-type 1980. The disease symptoms on the leaves of L16 and L20 were smaller than those on the control line (CK, transgenic line containing the empty vector). Specifically, the average lesion area on L16 and L20 was found to be 0.53 and 0.59 cm^2, respectively. These values represent a significant decrease of 47.5 and 42.2% compared to the control line, which had an average lesion area of 1.02 cm^2 (Fig. 4-A and B). Furthermore, the relative mRNA level of *SsBMR1* at 12 hpi was significantly suppressed in L16 and L20, which was 0.50 and 0.55 that of control line, specifically (Fig. 4-C). These findings demonstrate that in transgenic plants, the expression of *SsBMR1* is effectively downregulated, leading to impaired and delayed *S. sclerotiorum* infection.

3.5. *SsBMR1* is involved in resistance to toxin stress

To gain deeply understanding of the impact of *SsBMR1* on fungal virulence, two genotypes (Col-0 and L16) of *A. thaliana* plants were infected with two genotypes of *S. sclerotiorum* (1980 and Sibmr1-1) strains. As expected, the Sibmr1-1 exhibited the most significant reduction in lesion area on the L16 genotype, with approximately 58.7% reduced lesion area, compared to that 1980 on the Col-0 plants (Fig. 5-A and B). RNA-seq analysis was then employed to examine the global gene expression profile of *S. sclerotiorum* strains (1980 and Sibmr1-1) when inoculated onto different genotypes of *A. thaliana* (Col-0 and L16) at 12 hpi. DEGs among the comparison between WW (1980 inoculated on Col-0) and SiW (Sibmr1-1 inoculated on Col-0), WW and WH (1980 inoculated on L16) and WW and SiH (Sibmr1-1 inoculated on L16) were identified (Fig. 5-C; Appendix F). Functional enrichment analysis, using FungiFun, was performed on the 1,234 down-regulated DEGs across the three

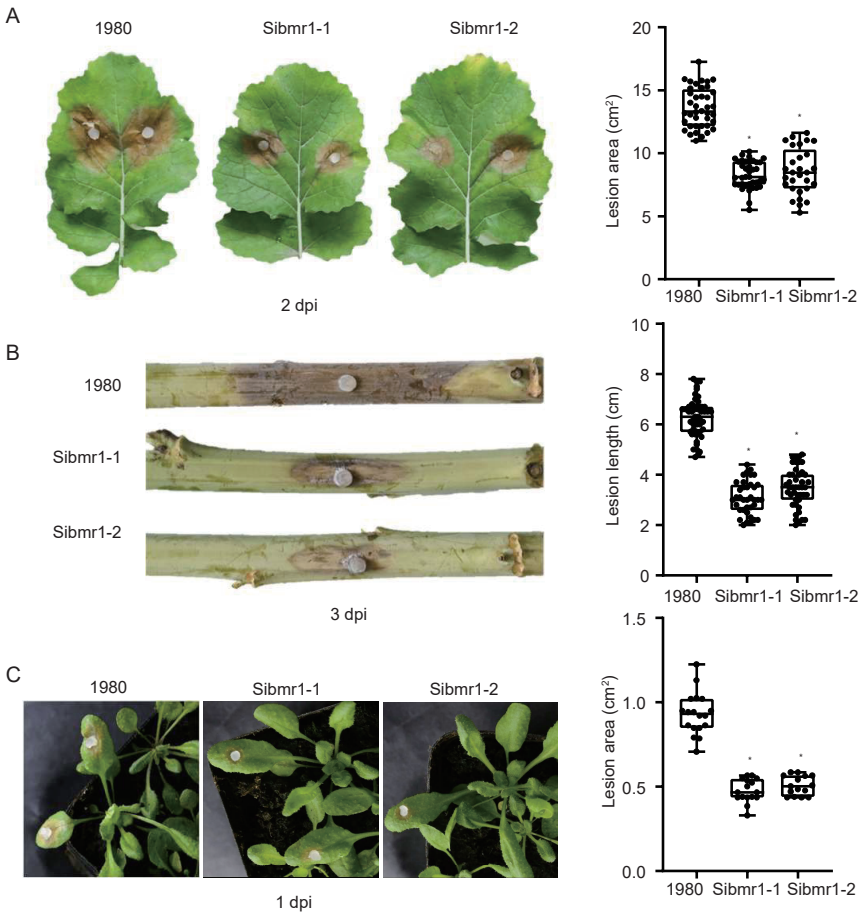


Fig. 3 Pathogenicity assays of the *SsBMR1* gene-silenced strains. Disease symptoms and disease quantification (lesion area or lesion length) on detached *Brassica napus* cv. Zhongshuang11 leaves (A), stems (B), and intact *Arabidopsis thaliana* leaves (C) caused by wild-type 1980, two *SsBMR1* gene-silenced transformants Sibmr1-1 and Sibmr1-2. Representative leaves were photographed. The data represent mean±SD (n>15 biological replicates). Asterisks (*) indicate a significant difference from 1980 at a P-value<0.05, determined by two-tailed t-test.

intercomparisons. The result revealed an overrepresentation of genes related to metabolism (such as secondary metabolism, metabolism of phenylalanine), cellular transport, transport facilitation and transport routes (including drug/toxin transport, heavy metal ion transport (Cu^{+}, Fe^{3+}, etc.), and peptide transport), as well as cell rescue, defense, and virulence (including detoxification by export and detoxification) among the whole down-regulated DEGs (Fig. 5-D). These findings suggest that silencing *SsBMR1* compromises fungal cell rescue, defense, and toxin transport facilitation.

We then evaluated the tolerance of *SsBMR1* gene-silenced transformants to the plant antifungal substances and fungicides. Both wild-type 1980 and *SsBMR1* gene-silenced transformants were inoculated on PDA medium supplementing with camalexin or brassinin (Fig. 6-A). The growth inhibition of Sibmr1-1 and Sibmr1-2 on camalexin was 55.9 and 54.9%, respectively, and on brassinin was 56.6 and 54.5%, respectively, which was significantly higher than that of 1980 (Fig. 6-B). In contrast, 1980 exhibited a growth inhibition of 31.3% on camalexin and 32.2% on brassinin. Furthermore, the virulence of *SsBMR1* gene-silenced transformants much more reduced on the leaves pre-incubated with camalexin or brassinin. The average lesion area of *SsBMR1* gene-silenced transformant Sibmr1-1 was reduced by 35.8 and 39.0%, whereas, the average lesion area of 1980 was reduced by 21.5 and 24.5% on the leaves pre-incubated with camalexin or brassinin, comparing to that treated with ddH_2O (Fig. 6-C and D).

The role of *SsBMR1* on tolerance to fungicides was further assessed. *SsBMR1* gene-silenced transformants exhibited significantly greater growth inhibition than 1980 when exposed to fungicides Boscalid and Azoxystrobin (Appendix G). However, there were no significant differences in the inhibition of hyphal growth between the *SsBMR1* gene-silenced transformants and the wild-type 1980 when treated with Triazolone and Carbendazim. These findings suggest that *SsBMR1* is involved in resistance to plant antifungal substances as well as certain fungicides.

3.6. *SsBMR1* is involved in handling extracellularly oxidative stress

ABC transporters can transport not only various xenobiotics but also natural metabolites (Víglaš and Olejníková 2021). We then performed a metabolomics analysis among wild-type 1980 and *SsBMR1* gene-silenced transformant Sibmr1-1 during the hyphae development.

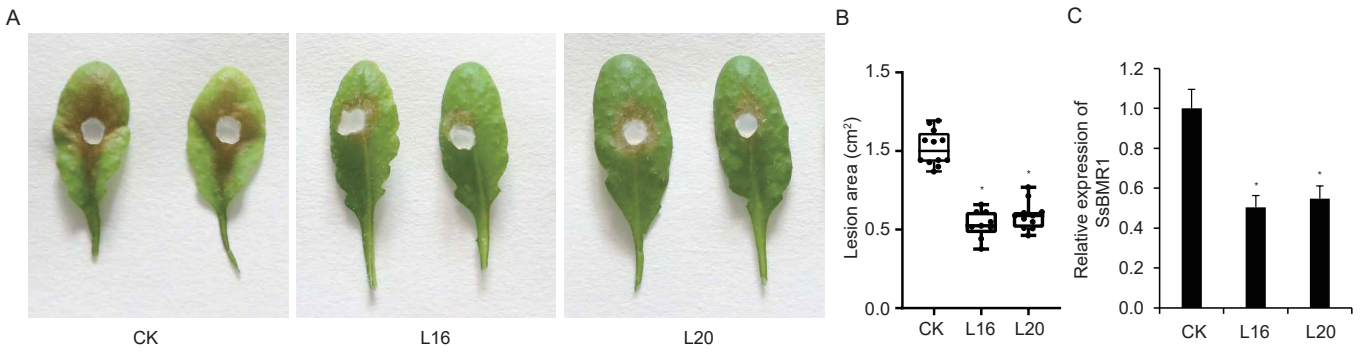


Fig. 4 Functional assessment of *SsBMR1* in *Sclerotinia sclerotiorum* pathogenicity determined by HIGS in *Arabidopsis thaliana*. Disease symptoms (A) and disease quantification (B) of CK (empty vector containing line), HIGS-*SsBMR1* transgenic *A. thaliana* lines (L16 and L20) at 48 hours post inoculation (hpi) with *S. sclerotiorum* strain 1980. Representative leaves were photographed at 48 hpi. C, relative transcript levels of *SsBMR1* in the leaves at 12 hpi with the *S. sclerotiorum* wild-type 1980. The quantity of *Sstub1* was used to normalize the expression levels of *SsBMR1* in different samples. The relative abundance of *SsBMR1* in the CK line was set as the control. The data in (B) represent mean±SD (n>15 biological replicates), and data in (C) represent mean±SD (n=3 biological replicates). Asterisks (*) indicate a significant difference from CK at a P-value<0.05, determined by two-tailed t-test.

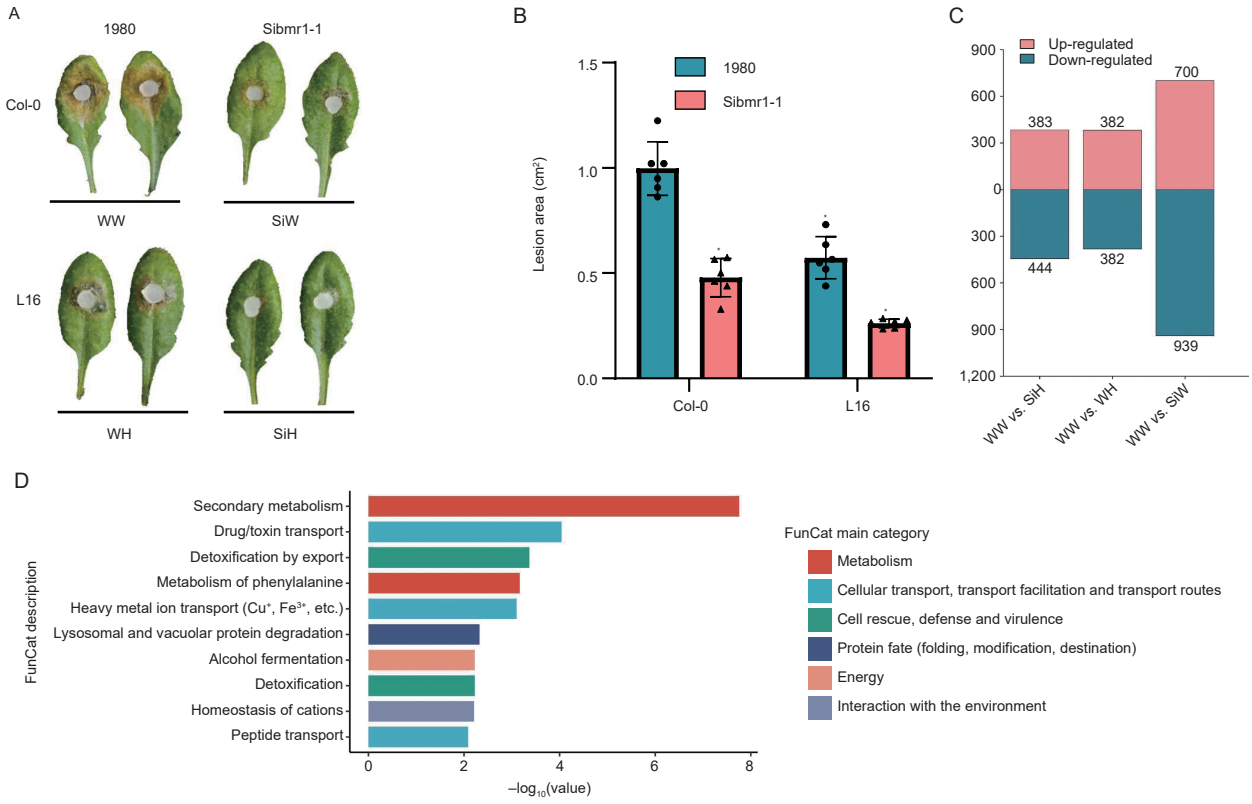


Fig. 5 RNA-seq analysis conducted on different transgenic lines infected with various strains. A, *Arabidopsis thaliana* wild-type Col-0 and the HIGS-*SsBMR1* transgenic *A. thaliana* line (L16) were challenged with *Sclerotinia sclerotiorum* wild-type 1980 and the *SsBMR1* gene-silenced transformant Sibmr1-1. Representative leaves were photographed 24 hours post inoculation (hpi). The experiment was performed three times and yielded consistent results. WW, 1980 inoculated on Col-0; SiW, Sibmr1-1 inoculated on Col-0; WH, 1980 inoculated on L16; SiH, Sibmr1-1 inoculated on L16. B, lesion area caused by strains 1980 and Sibmr1-1 on Col-0 and L16 at 24 hpi. The data represent mean±SD (n=6 biological replicates). Asterisks (*) indicate that the treatment means are significantly different from the wild type Col-0 at a P-value of <0.05, as determined by two-tailed t-tests. C, number of differentially expressed genes (DEGs) in *S. sclerotiorum*, comparing WW *vs*. WH, WW *vs.* SiW, and WW *vs.* SiH at 12 hpi. D, top ten significantly enriched FunCat terms for all down-regulated *S. sclerotiorum* DEGs in the comparisons WW *vs*. WH, WW *vs*. SiW, and WW *vs*. SiH.

1980 and Sibmr1-1 were cultured on the PDA plates, the mycelia were collected at 2, 7 and 15 days. A total of 115 metabolites were identified, covering 8 categories, including 46 organic acids and derivatives, 28 lipids and lipid-like molecules, 12 organic oxygen compounds, 11 nucleosides, nucleotides, and analogues, 7 benzenoids, 6

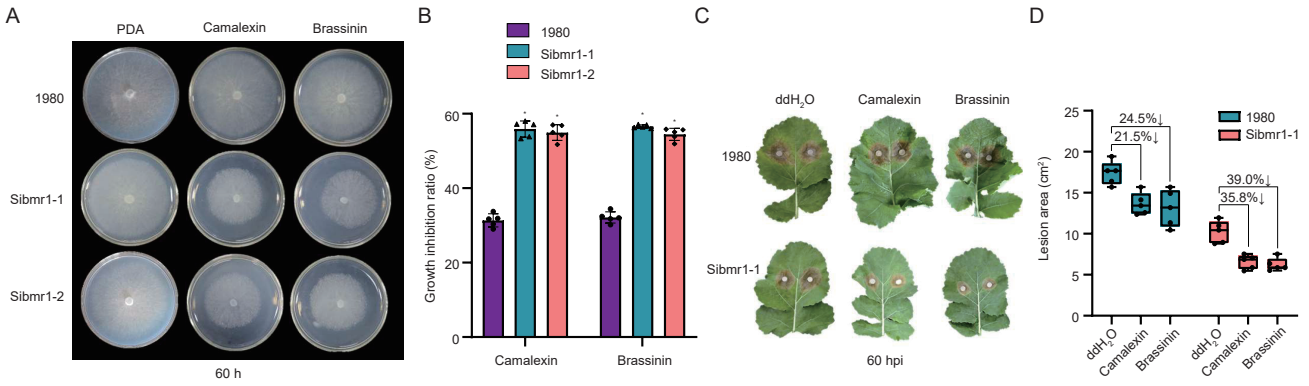


Fig. 6 Effects of *SsBMR1* on plant antifungal substances, camalexin and brassinin. Mycelium phenotypes (A) and growth inhibition (B) of wild-type strain 1980 and *SsBMR1* gene-silenced transformants grow on PDA supplemented with camalexin and brassinin. Disease symptoms (C) and lesion area (D) produced by 1980 and *SsBMR1* gene-silenced transformants on detached *Brassica napus* cv. Zhongshuang 11 leaves pre-incubated with ddH_2O, camalexin or brassinin for 30 min. One representative biological replicate is shown. The data represent mean±SD (n=5 biological replicates). Asterisks (*) indicate that the treatment means are significantly different from 1980 at a P-value of <0.05, as determined by two-tailed t-tests. hpi, hours post inoculation.

organoheterocyclic compounds, 4 phenylpropanoids and polyketides, and 1 alkaloids and derivatives (Appendix H). Differentially accumulated metabolites (DAMs) between 1980 and Sibmr1-1 were identified based on the thresholds |$\log_2$(fold change)|≥1 and VIP≥1. There were 22 DAMs between 1980 *vs*. Sibmr1-1 at 2 days; 33 DAMs at 7 days and 60 DAMs at 15 days (Fig. 7-A; Appendix I), with overlap of four DAMs, glutathione, divarinol, glutathione (reduced), and hippurate (Fig. 7-A and B). All of these four DAMs showed increased levels in the Sibmr1-1 at 2, 7 and 15 days, compared with 1980 (Fig. 7-B). Glutathione and glutathione (reduced) were involved in redox signaling through the removal of H_2O_2 that plays an important role in the oxidative defense (Dickinson and Forman 2002). We subsequently investigated the glutathione content in the mycelium and culture medium of 1980 and Sibmr1-1 and Sibmr1-2 after one day of cultivation in PDB medium containing 4 mmol L^{-1} H_2O_2. Compared to 1980, the mycelium of Sibmr1-1 and Sibmr1-2 exhibited a significant increase in glutathione level, while the culture medium showed a significant decrease (Fig. 7-C and D). It seems to imply that *SsBMR1* gene-silenced transformants were impaired in exporting the antioxidants.

We then assessed the response of *SsBMR1* in *S. sclerotiorum* under oxidative stress. qRT-PCR analysis revealed a significant increase in *SsBMR1* expression in *S. sclerotiorum* treated with H_2O_2 (Fig. 8-A). Subsequently, we examined the oxidative tolerance of *SsBMR1* gene-silenced transformants and wild-type 1980. The strains were cultured on PDA plates supplemented with varying concentrations of H_2O_2 (4, 8, and 12 mmol L^{-1}) (Fig. 8-B). The growth inhibition observed in 1980 on PDA plates supplemented with H_2O_2 was noticeably lower compared to Sibmr1-1 and Sibmr1-2 (Fig. 8-B and C). Furthermore, the mycelium of Sibmr1-1 and Sibmr1-2 displayed lower H_2O_2 content than 1980 (Fig. 8-D). These findings suggest that silencing *SsBMR1* impairs glutathione export and consequently compromises fungal detoxification of extracellular H_2O_2 in *S. sclerotiorum*.

4. Discussion

ABC transporters, integral to a superfamily of genes, encode membrane proteins that mediate drug efflux and substance transportation. These proteins are vital for fungal survival (Niimi *et al.* 2005). In this study, we demonstrated that the *S. sclerotiorum* ABC transporter gene *SsBMR1* is transcriptionally induced during host plant infection. Function analysis revealed that *SsBMR1* involves in the export of antioxidants and offers resistance to plant-derived antifungal substances, specifically camalexin and Brassinin, as well as certain fungicides. Knockdown of *SsBMR1* leads to a significant attenuation in virulence on both rapeseed and *A. thaliana*. Overall, our findings underscore the role of *SsBMR1* in virulence by facilitating the export of antioxidants and efflux of antifungal compounds.

SsBMR1 exhibits a (TMD-NBD)2 topology and is classified as an ABCG/PDR protein according to Víglaš and Olejníková (2021). ABCG proteins are referred to as pleiotropic drug resistance transporters. It has been observed that individual ABCG/PDR protein often exhibit activity against a range of chemically distinct compounds (Roohparvar *et al.* 2007). For instance, *N. crassa* CDR4, one of the four Pdr5p-like ABC transporters, has been identified as the major contributor to azole resistance (Zhang *et al.* 2012). In *S. cerevisiae*, Pdr5 is required for cantharidin resistance (Wawrzycka 2011; Swagatika and Tomar 2021). In *Sclerotinia homoeocarpa*, overexpression of the PDR transporter protein genes, *ShatrD* or *ShPDR1*, has been shown to confer increased resistance to certain fungicides such as propiconazole, iprodione, and pyrithiobac sodium (Hulvey *et al.* 2012; Sang *et al.* 2015). In this study, RNA-seq analysis suggested that silencing *SsBMR1* in *S. sclerotiorum* weakens fungal detoxification by inhibiting export and drug/toxin transport pathways. Notably, transformants with silenced *SsBMR1* exhibit increased sensitivity to the fungicides Boscalid and Azoxystrobin.

Fungal necrotrophs frequently encounter a plethora of toxic metabolites generated by the host, necessitating strategies for toxin transport and sequestration (Westrick

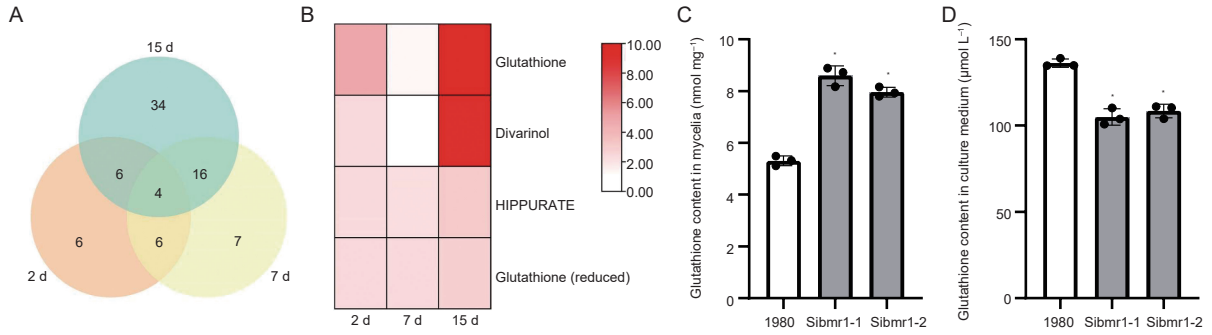


Fig. 7 Effects of *SsBMR1* on glutathione export. A, Venn diagram of differentially accumulated metabolites (DAMs) shared among two or three comparisons of wild-type 1980 *vs*. *SsBMR1* gene-silenced transformant Sibmr1-1 at 2, 7 and 15 days, respectively. B, heat maps of the overlapped 4 differentially accumulated metabolites (DAMs). C, glutathione (GSH) content in mycelia after one day of cultivation in PDB medium containing 4 mmol L^{-1} H_2O_2. D, GSH content in culture medium after one day of cultivation in PDB medium containing 4 mmol L^{-1} H_2O_2. The data in (C) and (D) represent mean±SD (n=3 biological replicates). Asterisks (*) indicate that the treatment means are significantly different from 1980 at a P-value of <0.05, as determined by two-tailed t-tests.

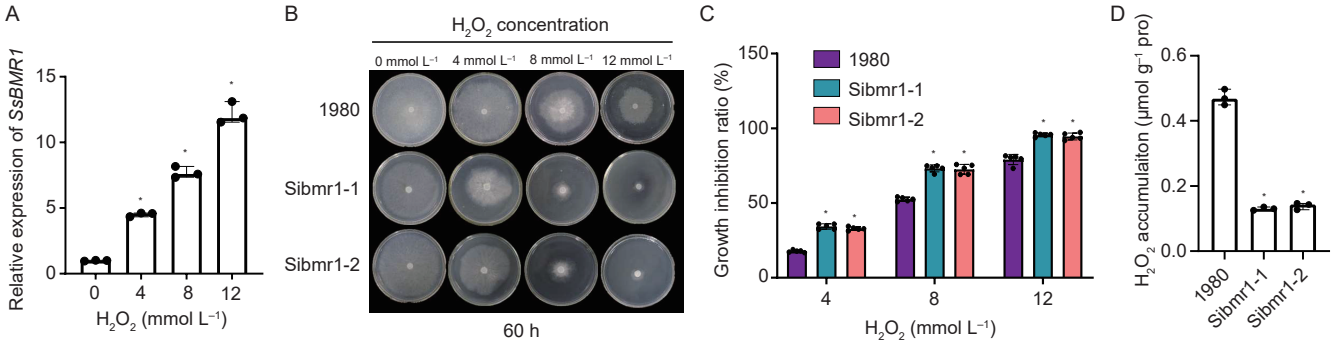


Fig. 8 Effects of *SsBMR1* on oxidative tolerance in *Sclerotinia sclerotiorum*. A, relative expression level of SsBMR1 when grown on PDA plates supplemented with different concentrations of H_2O_2 (4, 8, and 12 mmol L^{-1}). The expression levels were normalized using the *SsTub1* gene as an internal control, with the relative expression of *SsBMR1* on PDA plates supplemented with 0 mmol L^{-1} H_2O_2 set as one. B, phenotypes of wild-type 1980 and *SsBMR1* gene-silenced transformants when grow on PDA supplemented with different concentrations of H_2O_2 (4, 8, and 12 mmol L^{-1}). A representative biological replicate from five biological replicates is shown. C, growth inhibition of the wild-type 1980 and *SsBMR1* gene-silenced transformants on PDA supplemented with different concentrations of H_2O_2 (4, 8, and 12 mmol L^{-1}). D, quantitative of H_2O_2 level in the 2-day-old mycelia of wild-type 1980 and *SsBMR1* gene-silenced transformants. The data in (A), and (D) represent mean±SD (n=3 biological replicates), data in (C) represent mean±SD (n=5 biological replicates). Asterisks (*) indicate that the treatment means are significantly different from 1980 at a P-value of <0.05, as determined by two-tailed t-tests.

et al. 2021). Previous reports have demonstrated a significant upregulation of ABC transporter superfamily genes during infection (Seifbarghi *et al.* 2017; Westrick *et al.* 2019), consistent with our findings regarding the early induced expression of *SsBMR1* in *S. sclerotiorum* during various plant infections. These results suggest that *SsBMR1* has a certain role during infection. *SsBMR1* gene-silenced transformants were further found to exhibit increased resistance to the plant-derived antifungal compounds camalexin and brassinin. *B. cinerea* BcatrB also functions in exporting camalexin (Stefanato *et al.* 2009). Additionally, the ABCG protein AbcCl1 in *Colletotrichum lindemuthianum* was found to be involved in fungal resistance to toxic compounds produced by plants during common bean infections (Oliveira *et al.* 2022). Moreover, knockout of MgAtr5 in *Magnaporthe grisea* results in increased sensitivity to wheat phytoalexins and weakened pathogenic ability (Zwiers *et al*. 2002). Therefore, our findings highlight the crucial role of *SsBMR1* in fungal drug resistance and the efflux of antifungal agents during infection.

In addition to being able to transport antifungals, ABC transporters can also serve as the efflux of natural metabolites. *Sclerotinia cerevisiae* Adp1 was homolog to human ABCG2, can act as a glutathione exporter, which improved the extracellular glutathione production (Kiriyama *et al.* 2012). Glutathione plays a crucial role in scavenging ROS, antioxidation, and elimination of electrophiles (Liu *et al.* 2022). In yeast, glutathione serves an extracellular defence function to decrease toxicity (Thorsen *et al.* 2012). Yeasts secrete and accumulate glutathione extracellularly to eliminate harmful chemicals and thrive at high temperatures (Laman Trip and Youk 2020). Enhanced export of glutathione contributes to the resistance of astrocytes against copper toxicity (Scheiber and Dringen 2011). Impaired glutathione export and heightened sensitivity to extracellular H_2O_2 were observed in *SsBMR1* gene-silenced transformants, indicating *SsBMR1*'s potential role in facilitating antioxidant export to

counter host-derived ROS during infection.

Contrarily, ROS plays a crucial role in fungal growth and development during disease establishment and host infection. ROS impact fungal spore germination, cell differentiation, and the formation of infection structures and invasive hyphae (Zhang *et al.* 2020; Singh *et al.* 2021). In our study, we observed that *SsBMR1* gene-silenced transformants exhibited a higher glutathione content and lower ROS level in the fungal mycelium. These silenced transformants demonstrated impaired hyphal growth and reduced formation of infection cushions. Disruption of the ABC transporter MgAtr4 in *M. graminicola* led to inefficient colonization and intercellular growth in wheat leaves, while it did not affect *in vitro* growth (Stergiopoulos *et al.* 2003). *Magnaporthe grisea* ABC transporters ABC3 and ABC4 were found to influence appressoria formation in the early stages of infection (Sun *et al.* 2006; Gupta and Chattoo 2008). These results suggest the involvement of fungal ABC transporter genes in host establishment. Furthermore, we observed a reduction in sclerotia formation in *SsBMR1* gene-silenced strains. The possible reason may be that a promoting effect of ROS on sclerotia development in fungi (Kim *et al.* 2011; Wang *et al.* 2018; Zhang *et al.* 2020).

Developing inhibitors to modulate the activity of human ABC efflux pumps has emerged as a vital strategy in overcoming multidrug resistance in cancer (Ishikawa *et al.* 2012; Engle and Kumar 2022). Inhibiting ABCB1 and ABCG2 in Huh7 and PLC/PRF/5 cancer stem cells enhances their sensitivity to doxorubicin and reverses multidrug resistance (Yin *et al.* 2021). Our findings establish the crucial role of *SsBMR1* in virulence, as it fosters the export of antioxidants and expels antifungal compounds. Consequently, this present investigation paves the way for developing potential inhibitors against *SsBMR1*, which could be instrumental in combatting the formidable pathogen, *S. sclerotiorum*. Additionally, transgenic plants expressing HIGS-*SsBMR1* exhibited increased resistance to *S. sclerotiorum*. This aligns with a growing corpus of studies that have successfully generated resistant plant materials by inducing the silencing of various virulence genes through HIGS approaches (Wu *et al.* 2022; Wytinck *et al.* 2022; Pant and Kaur 2024; Tian *et al.* 2024; Xu *et al.* 2024). By aggregating these HIGS-derived materials, it is possible to develop highly resistant lines against Sclerotinia stem rot for the disease-resistant crop breeding. This research not only contributes valuable insights into the pathogenesis of *S. sclerotiorum* but also provides the groundwork for designing targeted interventions against this pathogen.

5. Conclusion

Our research reveals that *SsBMR1* is involved in the hyphal growth, infection cushion development, sclerotia formation, and virulence of *S. sclerotiorum*. *SsBMR1* plays a central role in the release of the antioxidant glutathione to confer resistance to extracellular oxidative stress. Furthermore, *SsBMR1* promoting resistance to plant antifungal substances including camalexin, brassinin, and certain fungicides, thereby contributing to the overall pathogenicity of *S. sclerotiorum*. The integrated understanding gained from this study opens up new possibilities for targeted interventions and control measures against this devastating plant disease.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used ChatGPT 4.0 to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

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