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RESEARCH ARTICLE

A GATA transcription factor contributes to multidrug resistance and pathogenicity through mediating the transcription of hydrolases and xenobiotic detoxification genes in *Sclerotinia sclerotiorum*

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Highlights

- A GATA transcription factor, SsGATA1, contributes to multidrug resistance by regulating the transcription of drug efflux pump genes.
- SsGATA1 positively regulates pathogenicity, which is attributed to the upregulation of hydrolases during infection.
- SsGATA1 represents a new target for preventing and controlling Sclerotinia stem rot.

Abstract

Phytopathogenic fungi can weaken the effectiveness of antifungal chemicals from plants and artificial synthesis through a xenobiotic detoxification system. Nevertheless, the transcription factors responsible for transcriptional activation of xenobiotic detoxification genes in phytopathogenic fungi are rarely reported. Here, we show that a GATA transcription factor, SsGATA1, regulates the transcription of drug efflux pump genes, thus contributing to tolerance to various types of chemical fungicides, including propiconazole, caspofungin, and azoxystrobin in *Sclerotinia sclerotiorum*. Similarly, SsGATA1 also confers tolerance to isothiocyanate and flavonols, two compounds reported as broad-spectrum antifungal chemicals, by mediating the transcription of the isothiocyanate hydrolase *SsSaxA*. Importantly, SsGATA1 positively regulates pathogenicity, which is attributed to the upregulation of hydrolases and *SsSaxA* during infection. Furthermore, SsGATA1 is responsible for tolerance to several stresses. Our findings demonstrate that SsGATA1 plays roles in multidrug resistance and pathogenicity by activating the transcription of hydrolases and xenobiotic detoxification genes.

Keywords: *Sclerotinia sclerotiorum*, multidrug resistance, GATA transcription factor SsGATA1, transcription, pathogenicity

1. Introduction

Phytopathogenic fungi pose serious threats to agricultural production. It is roughly estimated that diseases in the five most important crops (namely, rice, soybean, maize, potato, and wheat) may threaten ~60% of the world's grain output (Fones *et al.* 2020). Currently, fungal diseases are mainly controlled by breeding resistant cultivars and using chemical fungicides (Steinberg and Gurr 2020). The latter is still the most potent weapon when diseases occur, despite the rapid emergence of drug resistance (Steinhauer *et al.* 2019). The widely used fungicides, such as azoles, have a market of 3.3–4 billion US dollars (Jørgensen and Heick 2021). Although these chemical fungicides may have different mechanisms of action, they can enter fungal cells, target specific molecular structures or indiscriminately attack enzymes that are critical to the survival of pathogenic fungi, and eventually inhibit fungal growth or even kill them directly (Odds *et al.* 2003; Schuster *et al.* 2024). In order to resist or eliminate the cell damage caused by fungicides, pathogenic fungi, on the one hand, show drug resistance by changing the target to

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escape the attack of fungicides (Fisher *et al.* 2018; Berman and Krysan 2020; Osset-Trénor *et al.* 2023), and on the other hand, can reduce the accumulation of fungicides themselves or toxic substances produced by them through catabolism and drug efflux pumps, thereby increasing the dosage required and often exhibiting multiple drug resistance (MDR) (de Waard *et al.* 2006; Gulshan and Moye-Rowley 2007; Berman and Krysan 2020; Osset-Trénor *et al.* 2023). The latter is usually related to the fact that pathogens can tolerate fungicides below the minimum inhibitory concentration (MIC) (Berman and Krysan 2020).

MDR refers to the resistance of pathogens to a variety of fungicides with different modes of action, which is often related to the activation, expression, and activity improvement of ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters (Gulshan and Moye-Rowley 2007; Berman and Krysan 2020; Osset-Trénor *et al.* 2023). Fungi allocate a large part of their genome resources to encode these transporters, and the roles of ABC and MFS in drug efflux have been widely studied, especially in *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida glabrata* (Sipos and Kuchler 2006; Ren *et al.* 2007; Drew *et al.* 2021; Víglaš and Olejníková 2021). *Candida albicans* can overexpress the ABC transporters CDR1 and CDR2 or the MFS transporter MDR1, which leads to increased drug efflux activity and resistance to azole drugs, and also confers resistance to other antifungal drugs (Sastré-Velásquez *et al.* 2022; Osset-Trénor *et al.* 2023). Even the overexpression of a single transporter caused by adaptive mutation can lead to increased drug efflux ability, thus reducing sensitivity to multiple drugs (Gulshan and Moye-Rowley 2007). ABC and MFS transporters are also known to be involved in the efflux of natural and synthetic drugs in plant pathogenic fungi (de Waard *et al.* 2006). For example, among 14 ABC transporters (BcatrA to BcatrN) of *Botrytis cinerea*, at least *BcatrB*, *BcatrD*, and *BcatrK* genes are reported to be directly related to MDR (Vermeulen *et al.* 2001; Kretschmer *et al.* 2009; Wu *et al.* 2024). The study of gene knockout revealed that AtrB of *B. cinerea* can transport a variety of fungicides and antifungal compounds from plants and microorganisms (Vermeulen *et al.* 2001). When tested, resistant *B. cinerea* strains showed lower fungicide accumulation than sensitive strains, indicating that the resistant strains had higher efflux activity, which was attributed to the overexpression of ABC transporter AtrB (Vermeulen *et al.* 2001; Kretschmer *et al.* 2009). AtrB, as well as its homologues in other filamentous fungi, is a conserved drug efflux pump that can contribute to tolerance to various fungicides and natural antifungal compounds (de Waard *et al.* 2006; Stefanato *et al.* 2009; Wu *et al.* 2024). The MFS transporters also mediate the efflux of fungicides and plant- or microbial-derived antifungal compounds (Hayashi *et al.* 2002). Correspondingly, several drug efflux transporter genes are rapidly induced by fungicides or natural antifungal compounds, such as *atrD* and *atrB* of *B. cinerea* as well as *Atr1* and *Atr2* of *Mycosphaerella graminicola* (Vermeulen *et al.* 2001). Although a consensus has been reached on the mechanism by which pathogenic fungi develop MDR through up-regulating these drug efflux pumps, the transcription factors responsible for transcriptional activation of drug efflux pump expression have been functionally characterized only in a few pathogenic fungi (Vermitsky and Edlind 2004; MacPherson *et al.* 2006; Thakur *et al.* 2008; Kretschmer *et al.* 2009; Lohberger *et al.* 2014; Rybak *et al.* 2020; Osset-Trénor *et al.* 2023), but remain unclear in plant pathogenic fungi. Thus, identifying new transcription factors responsible for regulating the transcription of drug efflux pump genes will help reveal the molecular mechanism of MDR in plant pathogenic fungi and facilitate the development of new fungicides targeting these transcription factors.

In addition to synthetic chemical fungicides, plants can also use their own secondary metabolites as chemical defense weapons (Flórez *et al.* 2017), such as glucosinolates produced by Brassicales that generate toxic isothiocyanates (ITCs), conferring broad resistance against plant pathogens (Bednarek *et al.* 2009; Chen *et al.* 2020). Flavonoids are multifunctional phenolic natural products ubiquitous in plants and also protect plants from infection by pathogens (Buer *et al.* 2010; Chen *et al.* 2019). Although plants can produce various chemical weapons to resist invading insects and pathogens, many pathogens, such as *Sclerotinia sclerotiorum*, can still overcome this chemical defense and infect hosts rich in these flavonoids or ITCs (Chen *et al.* 2019, 2020). *Sclerotinia sclerotiorum* is an aggressive necrotrophic plant pathogen with a wide host range, including more than 600 species, causing substantial economic losses in many important agricultural crops, such as flavonoid-rich soybean and oilseed rape that produce ITCs (Bolton *et al.* 2006; Shang *et al.* 2024). Current reports have confirmed that *S. sclerotiorum* can degrade toxic flavonoids and ITCs through a quercetin dioxygenase (SsQDO) and an ITCase (SsSaxA), respectively, thereby circumventing the chemical defense mechanisms of plant flavonoids and ITCs and ensuring effective infection (Chen *et al.* 2019, 2020). Compared with the wild type, strains with *SsQDO* or *SsSaxA* knocked out grow slowly in media containing flavonoids or ITCs, and their infection of *Arabidopsis thaliana* is also reduced. It is worth noting that the expression level of *SsSaxA* is minimal in the absence of ITCs and rises rapidly after induction by ITCs (Chen *et al.* 2020), indicating that *S. sclerotiorum* can perceive ITCs in the environment or host and activate *SsSaxA* expression to degrade ITCs through an unknown transcriptional mechanism. Similarly, *SsQDO* is also up-regulated by flavonoids (Chen *et al.* 2019). However, the transcription factors responsible for these transcriptional activation processes remain unclear. Therefore, identifying the transcription factors that activate the transcription of enzymes degrading plant-derived toxic substances and blocking this transcriptional process may restore the chemical defense function of plants.

GATA transcription factors are zinc finger transcription factors that specifically bind to the A/TGATAA/G motif in DNA and are widely conserved in eukaryotes (Evans *et al.* 1988). In filamentous fungi, GATA transcription factors are reported to be involved in various biological processes, including morphogenesis, virulence, nutrient utilization, secondary metabolism, reactive oxygen species homeostasis regulation, and stress response (Macios *et al.* 2012; Marroquin-

Guzman and Wilson 2015; Sun *et al.* 2024). We previously identified nine GATA transcription factors in *S. sclerotiorum* based on GATA domain sequence similarity, including SsNsd1, which regulates sclerotium and infection cushion formation (Li *et al.* 2018); SsSfh1, responsible for chromatin remodeling and DNA repair (Liu *et al.* 2018a); and SsAms2, involved in chromosome segregation (Liu *et al.* 2018b). Here, we describe the function of the GATA transcription factor SsGATA1 in detail. The results show that SsGATA1 is not involved in mycelial growth, sclerotium formation, and infection cushion development in *S. sclerotiorum*, but participates in the pathogenic process by positively regulating the transcription of hydrolases and xenobiotic detoxification genes. Furthermore, SsGATA1 also acts as a transcriptional activator of xenobiotic detoxification genes to regulate MDR. Therefore, SsGATA1 may be a potential new target for controlling Sclerotinia stem rot (SSR).

2. Materials and methods

2.1. Fungal strains and culture conditions

The *S. sclerotiorum* wild type (WT) strain UF-1 was used as a parental strain. The WT and its derivative strains in this study were initially grown on potato dextrose agar (PDA) plates and cultured at 25°C.

2.2. Identification and sequence analysis of genes

The protein sequence of SsGATA1 (XP_001595686.1) was used as a query to perform a BLASTp search to obtain homologous protein sequences in several pathogenic fungi. The SsGATA1 phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates in MEGA 7.0. The predicted three-dimensional protein structure of SsGATA1 was obtained from the AlphaFold database (https://alphafold.ebi.ac.uk/entry/A7EEN5).

2.3. Gene deletion and genetic complementation

Gene deletion mutants for the *SsGATA1* gene of *S. sclerotiorum* were generated using a split-marker PCR-mediated homologous recombination strategy, and the protoplast transformation method was used (Rollins 2003; Jiao *et al.* 2022). Briefly, the flanking sequences FR1 and FR2 of *SsGATA1* were amplified from genomic DNA of the WT strain with GATA1-FP1/GATA1-RP1 and GATA1-FP2/GATA1-RP2, respectively, and the hygromycin phosphotransferase gene cassette was amplified from the pUCATPH vector to obtain overlapping HY and YG fragments using M13R/NLC37 and M13F/NLC38. Then, FR1/HY and FR2/YG were used as templates fused by GATA1-FP1/NLC37 and GATA1-RP2/NLC38 to obtain final knockout fragments. These fragments were used to transform protoplasts of the WT strain generated with lysing enzymes from *Trichoderma harzianum* (L1412, Sigma-Aldrich, St. Louis, USA) and introduced using the polyethylene glycol (PEG)-mediated transformation method. Transformants were purified by hygromycin selection (100 μg mL^{-1}, Sigma-Aldrich, St. Louis, USA), hyphal-tip purified at least five times, and then screened by PCR with primers M13F/M13R and Gene-F/R.

To complement the *SsGATA1* knockout mutants, the nucleotides including flanking sequences FR1 and the full-length *SsGATA1* gene were amplified from genomic DNA of the WT strain with FP1-C/RP1-C, the flanking sequence FR2 was amplified from genomic DNA with FP2-C/RP2-C, and the geneticin resistance cassette was amplified from the pII99 vector using DW69/DW70. These fragments were used to transform protoplasts of the *SsGATA1* knockout mutants according to the above transformation method. Transformants were purified using G418 selection (100 μg mL^{-1}, Sangon Biotech, Shanghai, China), hyphal-tip purified at least three times, and then screened by PCR with primers Gene-F/R. Subsequently, RNA was extracted from WT and transformants inoculated on PDA and covered with cellophane, respectively, and the expression level of *SsGATA1* was determined by qRT-PCR. All the primers are listed in Appendix A.

2.4. Analysis of hyphal growth, sclerotia, and infection cushions

To observe hyphal growth and sclerotia formation, the WT and its derivative strains were inoculated on PDA plates, incubated for 0–14 d at 25°C, and colony diameters were measured every 12 h. Sclerotia development was photographed, and quantity and dry weight were measured. At least three technical and three biological replicates were performed for each strain.

Glass slides were used as substrata to induce the formation of infection cushions. The formation of infection cushions on glass slides was observed at 12 hours post-inoculation (hpi) under a bright-field light microscope. ImageJ software was used to estimate the gray value of infection cushion images to quantify infection cushion biomass formed on glass slides at 24 hpi. At least nine technical and three biological replicates for each strain were performed.

2.5. RNA extraction and qRT-PCR

Samples for gene expression analysis are described in subsequent sections or refer to previous methods (Xiao *et al.* 2024). Total RNA purification, reverse transcription, and qRT-PCR were completed according to a one-step kit (TransGen, Beijing, China). The *S. sclerotiorum* β-tubulin gene *Sstublin* (SS1G_04652) or *Ssactin* (SS1G_08733) was used to normalize transcript levels. The relative expression levels were calculated with three technical replicates using the $2^{-\Delta\Delta CT}$ method. All primers used for qRT-PCR are listed in Appendix A.

2.6. Anti-fungal chemicals and stress treatments

For high temperature (HT, 30°C), oxidative (5 mmol L^{-1} H_2O_2, 5 mmol L^{-1} H_2O_2, and 30 μmol L^{-1} menadione), hyperosmotic (1 mol L^{-1} KCl and 1 mol L^{-1} sorbitol), reagents that disrupt cell wall integrity (500 μg mL^{-1} Congo red, 10 μg mL^{-1} calcium fluoride white, and 0.01% sodium dodecyl sulphate), and nutrient-limited (MM, MM-C, and MM-N) stress treatments, the methods followed those of a previous study (Xiao *et al.* 2024). Colony diameters were measured at 36 h, and growth inhibition ratios were calculated. The growth inhibition

ratio=(Colony diameter on untreated PDA−Colony diameter on treated PDA)/(Colony diameter on untreated PDA×100%) or (Colony diameter on complete medium−Colony diameter on nutrient-limited medium)/(Colony diameter on complete medium×100%).

For anti-fungal chemical treatments, PDA plates with propiconazole (0.05 μg mL^{-1}, HY-B0847, MCE), caspofungin (0.2 μg mL^{-1}, HY-17006, MCE, Shanghai, China), nikkomycin Z (1 μg mL^{-1}, HY-19593, MCE, Shanghai, China), myriocin (1 μg mL^{-1}, HY-N6798, MCE, Shanghai, China), 0.5 μg mL^{-1} azoxystrobin, 2PE-ITC (40 μmol L^{-1}, HY-23155, MCE, Shanghai, China), quercetin (0.02 mg mL^{-1}, HY-18085, MCE, Shanghai, China), or kaempferol (0.02 mg mL^{-1}, HY-14590, MCE, Shanghai, China) were used for culturing strains for 36 h. Colony diameters were measured at 36 h, and growth inhibition ratios were calculated. To analyze the expression of xenobiotic detoxification genes under anti-fungal chemical treatments, hyphae were cultured in PDB, treated with 0.05 μg mL^{-1} propiconazole or 40 μmol L^{-1} 2PE-ITC for 1 h, collected, and compared to hyphae cultured in PDB by qRT-PCR.

2.7. Yeast one-hybrid (Y1H) assays

The coding sequences of SsGATA1 and SsGATA1 with the GATA zinc finger domain removed were amplified from cDNA and cloned into pGADT7 to generate AD-SsGATA1 and $SsGATA1^{\Delta ZnF}$. The motif containing three GATA box motifs in series and a GATA box mutation were artificially synthesized, and the promoter sequence of target genes (approximately 800 bp) was amplified by PCR. Then all the fragments were cloned into the pAbAi vector and transformed into Y1HGold yeast competent cells after linearization by enzyme digestion. Subsequently, the pGADT7 clones were transformed, and yeast transformants were selected on synthetic dropout (SD)-Ura/Leu (Coolaber, Beijing, China) medium. The interaction between DNA and protein was tested on SD-Ura/Leu supplemented with a designated concentration of aureobasidin A (AbA) (CA2332, Coolaber, Beijing, China). All primers are listed in Appendix A.

2.8. Pathogenicity assays

To assess pathogenicity, 6-week-old soybean leaves, 5-week-old *Nicotiana benthamiana* leaves, and 5-week-old Arabidopsis Col-0 leaves were detached and placed in a 95% relative humidity box at 25°C. Mycelial plugs from the WT and its derivative strains grown on PDA were used for inoculation. The pathogenicity and virulence of the strains were evaluated according to the lesion area at 24 hpi. The lesion area was calculated using ImageJ. To demonstrate the expression changes of *SsSaxA* during infection, hyphae from 2-day PDB cultures were inoculated onto Arabidopsis leaves. Samples of hyphae and diseased spots on leaves were used for qRT-PCR at 24 hpi.

2.9. Statistical analyses

Statistical analyses were performed with Prism 8.0 software (GraphPad). Statistical analysis methods are described in the figure legends.

3. Results

3.1. SsGATA1 is a GATA transcription factor induced by stress

Our previous study found that *S. sclerotiorum* contains nine proteins with GATA zinc finger domains; among them, the protein encoded by *SS1G_03775* has also been identified as having a GATA domain, but its function is still unclear. Phylogenetic analysis showed that it was distant from other proteins except BCIN_04g03800, a putative protein of *B. cinerea* (Fig. 1-A). Conserved domain analysis and protein structure prediction identified that residues 450-478 constitute a classic GATA zinc finger domain (Fig. 1-B and C); hence, it is named SsGATA1 here. To prove that SsGATA1 is a GATA transcription factor, SsGATA1 and a version of SsGATA1 with the GATA zinc finger domain removed were fused with the activation domain of GAL4 and expressed in yeast, and Y1H experiments were carried out with a synthetic three -tandem GATA box motif. The screening results of the reporter gene showed that AD-SsGATA1 could bind to the GATA box but not to the mutated GATA box (mGATA); removal of the GATA zinc finger domain abolished the ability of SsGATA1 to bind the GATA box, and the activation domain (AD) alone did not interact with either (Fig. 1-D), suggesting that SsGATA1 is a transcription factor that binds the GATA box *via* its GATA zinc finger domain.

To determine which biological processes SsGATA1 plays a role in *S. sclerotiorum*, the expression level of *SsGATA1* was examined first. The qRT-PCR results showed that *SsGATA1* had a low expression level in the hyphal stage and was up-regulated in sclerotia, infection cushion stage, and early stage of infection. Moreover, expression was highly up-regulated after heat stress and oxidative stress induced by menadione or H_2O_2, especially after menadione treatment, which was nearly 400 times higher than that of untreated mycelium (Fig. 1-E). These results indicate that SsGATA1 may play a role in infection and stress response in *S. sclerotiorum*.

3.2. SsGATA1 is dispensable in the morphogenesis in *S. sclerotiorum*

To explore the biological function of SsGATA1 in *S. sclerotiorum*, we generated gene deletion mutants and genetic complementation strains for *SsGATA1* (Appendix B). When cultured on PDA plates for 2 days, no significant difference was observed between the Δ*SsGATA1* mutant and the WT or complementation strains (Δ*SsGATA1*-C) in hyphal growth rate and colony morphology (Fig. 2-A and B). Prolonging the culture time showed that SsGATA1 did not affect colony morphology, sclerotium formation, and sclerotium dry weight (Fig. 2-A and C). When infection cushions were induced on glass slides, the Δ*SsGATA1* mutant was indistinguishable from the WT and complementation strains in its ability to form infection cushions (Fig. 2-D and E). These results suggest that SsGATA1 is dispensable for morphogenesis in *S. sclerotiorum*.

3.3. SsGATA1 regulates the transcription of hydrolases to cause disease

The role of SsGATA1 in pathogenicity was also evaluated. When inoculated on the leaves of *A. thaliana*, *N. benthamiana*,

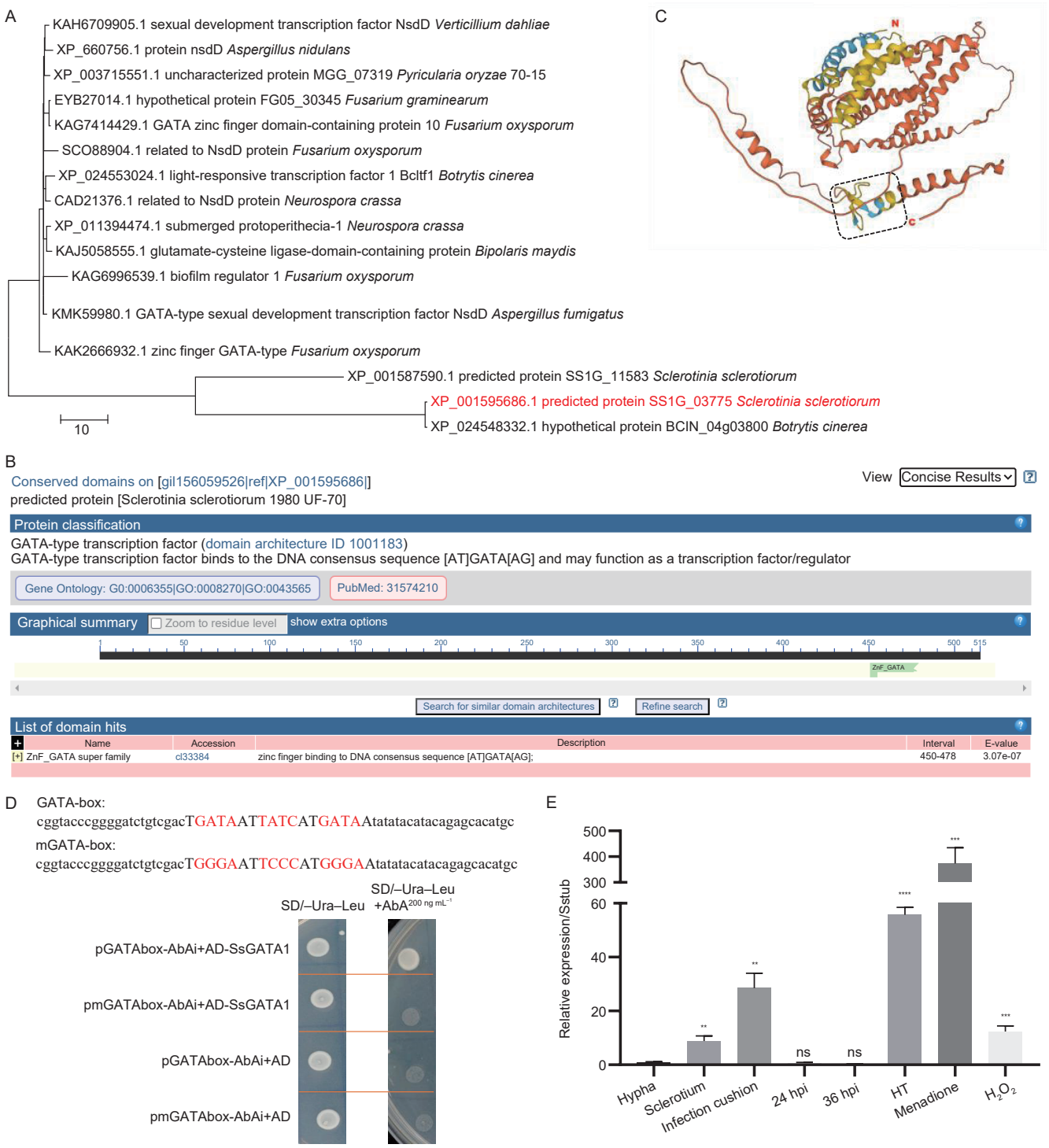


Fig. 1 Identification and transcription level analysis of GATA transcription factor SsGATA1. A, phylogenetic analysis of SsGATA1 and its homologs in fungi. The phylogenetic tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates in MEGA 7.0. B, SsGATA1 possesses a GATA zinc finger domain. The conserved domain of SsGATA1 was identified by the pipeline for identifying conserved domains in NCBI. C, protein structure of SsGATA1 was predicted by AlphaFold (https://alphafold.ebi.ac.uk/entry/A7EEN5). The GATA zinc finger domain is highlighted in the black dotted box. N, amino-terminus; C, carboxyl-terminus. D, SsGATA1 specifically binds to the GATA box motif in Y1H assays. At the top is the schematic representation of the synthetic three tandem GATA box motifs and the mutated version with the GATA box (mGATA). Red letters refer to GATA sequences. SD/–Ura–Leu, synthetic medium (SD) without uracil and leucine. AD, pGADT7; SsGATA1$^{\Delta ZnF}$, SsGATA1 with the GATA zinc finger domain removed. E, expression level of *SsGATA1* was determined by qRT-PCR in different stages of the life cycle, infection process, and under several stress conditions. Samples include hyphae, sclerotia, infection cushions, inoculated *Arabidopsis thaliana* at 12, 24, and 36 h post inoculation (hpi), and those treated with 30°C, 30 μmol L^{-1} menadione, or 5 mmol L^{-1} H_2O_2 for 1 h. HT, high temperature. *Sstublin* was used as an internal control, and the expression level of *SsGATA1* in hyphae was normalized to 1. Error bars represent mean±SD (n=3). Data were analyzed by two-tailed Student's t-test compared to hyphae, and asterisks represent significant differences (ns, no significance; **, P<0.01; ***, P<0.001; ****, P<0.0001).

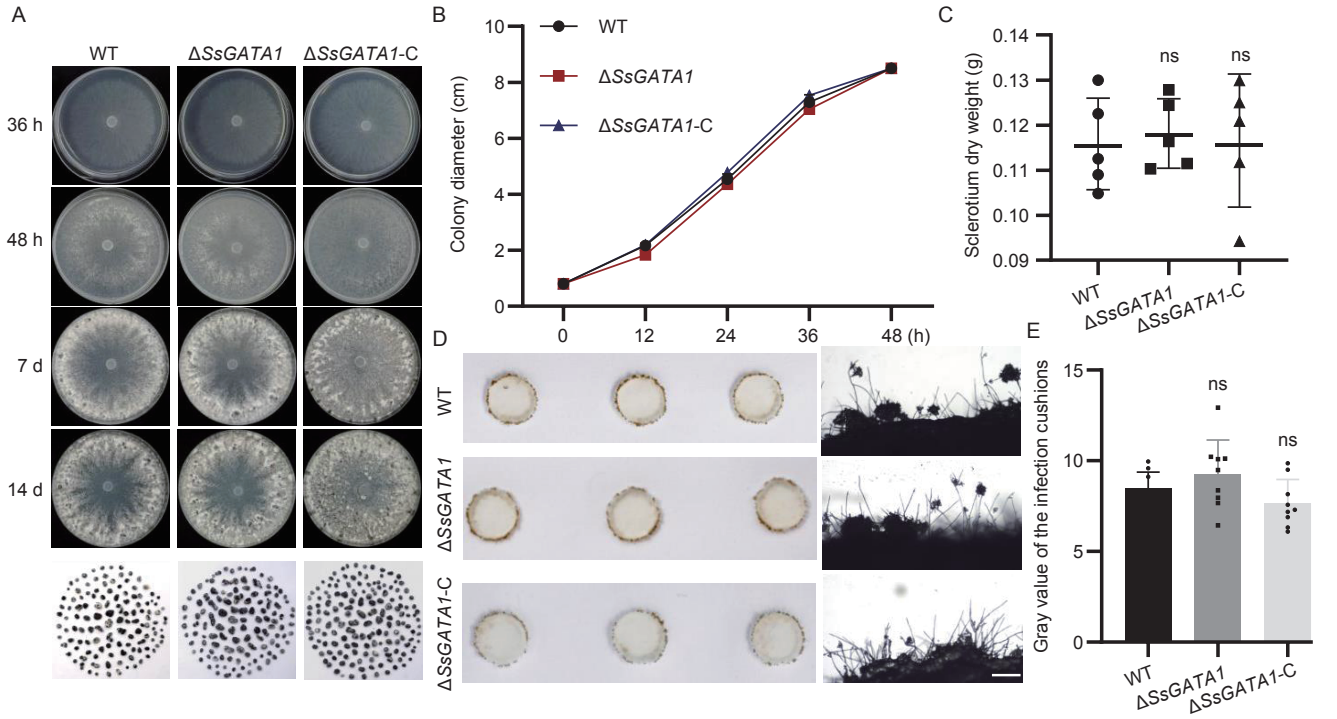


Fig. 2 SsGATA1 has no effect on morphogenesis in *Sclerotinia sclerotiorum*. A, effect of SsGATA1 deletion on hyphal growth, colony morphology, and sclerotium formation. Colony morphology of the WT, Δ*SsGATA1* mutant, and complementation strain Δ*SsGATA1*-C on PDA plates grown continuously for 2 weeks. The pictures were taken at 36 h, 48 h, 7 d, and 14 d post incubation. B, colony diameter of the WT, Δ*SsGATA1* mutant, and Δ*SsGATA1*-C on PDA plates was measured every 12 h. C, dry weight of sclerotia of the WT, Δ*SsGATA1* mutant, and Δ*SsGATA1*-C was weighed after 2 weeks on each PDA plate. All individual data points (*n*=5) are overlaid. D, infection cushion formation of the WT, Δ*SsGATA1* mutant, and Δ*SsGATA1*-C was induced by glass slides. Scale bar: 200 μm. E, the gray value representing the infection cushions was calculated. All individual data points (*n*=9) are overlaid. For C and E, data were analyzed by two-tailed Student's *t*-test compared to WT (ns, no significance).

and soybean *in vitro*, the lesion area caused by the Δ*SsGATA1* mutant was significantly smaller than that of the WT and Δ*SsGATA1*-C, indicating that the Δ*SsGATA1* mutant had lower virulence, especially in leaf maceration ability (Fig. 3-A and B).

Therefore, the expression levels of genes encoding various hydrolases in the WT, Δ*SsGATA1* mutant, and Δ*SsGATA1*-C were compared after inoculation on leaves of soybean, *A. thaliana*, or *N. benthamiana*. The qRT-PCR results (Fig. 3-C, D, and E) showed that the transcription levels of cutinases (*SsCut1/2*), glycosyl hydrolase 5 (cellulase) family (*SsGH5-1/2*), pectin lyases (*SsPL1/2*), extracellular lipase (*SsLip1*), and aminopeptidase (*SsPep1*) in the WT and Δ*SsGATA1*-C were acutely induced after inoculation for 48 or 24 h, and the induction levels of *SsCut1/2*, *SsGH5-1/2*, and *SsPL1/2* were significantly reduced in the Δ*SsGATA1* mutant, although the levels of *SsLip1* and *SsPep1* showed no significant difference among the Δ*SsGATA1* mutant, WT, and Δ*SsGATA1*-C. In addition, SsGATA1 did not affect the induced expression of the important necrosis-inducing factor *SsNEP2* during infection (Yang *et al.* 2022) (Fig. 3-C), suggesting that SsGATA1 specifically regulates the transcription of some hydrolases during infection. Importantly, Y1H confirmed that SsGATA1 can directly bind to the promoters of *SsCut1* and *SsPL1* (Fig. 3-F). Overall, our data demonstrate that SsGATA1, as a transcription factor, binds to the promoters of hydrolase genes to activate transcription and contribute to pathogenicity.

3.4. SsGATA1 mediates tolerance to multiple stresses

Given that *SsGATA1* is up-regulated under various stresses, the role of SsGATA1 in stress resistance in *S. sclerotiorum* was evaluated. Consistent with its transcriptional up-regulation under heat stress and oxidative stress, the Δ*SsGATA1* mutant was significantly more sensitive to heat stress than the WT and Δ*SsGATA1*-C. Similarly, the Δ*SsGATA1* mutant was significantly more sensitive to 5 mmol L^{-1} H_2O_2 or menadione than the WT and Δ*SsGATA1*-C, although growth in 10 mmol L^{-1} H_2O_2 was almost the same as that of the WT and Δ*SsGATA1*-C (Fig. 4-A and B). We also evaluated the growth of these strains with reagents that disrupt cell wall integrity. The results showed that the sensitivity of the Δ*SsGATA1* mutant to CFW, SDS, and CR was not significantly different from that of the WT and Δ*SsGATA1*-C. Likewise, the tolerance of the Δ*SsGATA1* mutant to two hyperosmotic stress inducers, KCl and sorbitol, was similar to that of the WT and Δ*SsGATA1*-C (Fig. 4-B and C). In addition, SsGATA1 is not involved in regulating growth under nutrient-limited conditions, as the growth of the Δ*SsGATA1* mutant on MM, MM-C, and MM-N was indistinguishable from that of the WT and Δ*SsGATA1*-C (Fig. 4-B and D). These results indicate that SsGATA1

regulates tolerance to heat stress and oxidative stress in *S. sclerotiorum*.

3.5. SsGATA1 contributes to multidrug resistance by positively regulating the transcription of drug efflux pump genes

The main problem hindering the control of SSR is that *S. sclerotiorum* exhibits tolerance to many fungicides, and drug resistance continues to emerge in the field, leading to the need for high fungicide dosages or even control failure. The most important class of agricultural fungicides are triazoles, which combine low cost, reliable performance, and broad effectiveness (Jørgensen and Heick 2021). Among them, propiconazole is widely used to control SSR; thus, we determined the role of SsGATA1 in propiconazole resistance. Plate assays showed that the Δ*SsGATA1* mutant was significantly more sensitive to propiconazole than the WT and Δ*SsGATA1*-C (Fig. 5-A and B). Subsequently, we also evaluated the effect of SsGATA1 on resistance to several drugs targeting fungal cell wall synthesis, including caspofungin, which inhibits 1,3-glucan synthesis (Hashemian *et al.* 2020); nikkomycin Z, which inhibits chitin synthesis (Chaudhary *et al.* 2013); and myriocin, which inhibits glycosylphosphatidylinositol-anchored protein and sphingolipid synthesis (Mota-Fernandes and Del-Poeta 2020). The results showed that the Δ*SsGATA1* mutant was also significantly more sensitive than the WT and Δ*SsGATA1*-C to these drugs (Fig. 5-A and B). Similarly, the Δ*SsGATA1* mutant was highly sensitive to the broad-spectrum fungicide azoxystrobin (Xu *et al.* 2013) (Fig. 5-A and B), suggesting that SsGATA1 regulates MDR.

MDR is usually associated with high activity or expression of drug efflux pumps; thus, the influence of SsGATA1 on the expression of genes encoding drug efflux pumps was analyzed. The qRT-PCR results revealed that although the expression levels of two MFS transporters and five ABC transporters in the Δ*SsGATA1* mutant were similar to those in the WT and Δ*SsGATA1*-C without propiconazole treatment, these genes were highly up-regulated in the WT and Δ*SsGATA1*-C after 1 h of propiconazole treatment, and this up-regulation was significantly weaker in the Δ*SsGATA1* mutant (Fig. 5-C). Subsequent Y1H experiments confirmed that SsGATA1 can directly bind to the promoters of *SsMFS1* and *SsATRD* to activate reporter gene expression (Fig. 5-D). In summary, these results suggest that SsGATA1 contributes to MDR by binding to the promoters of drug efflux pump

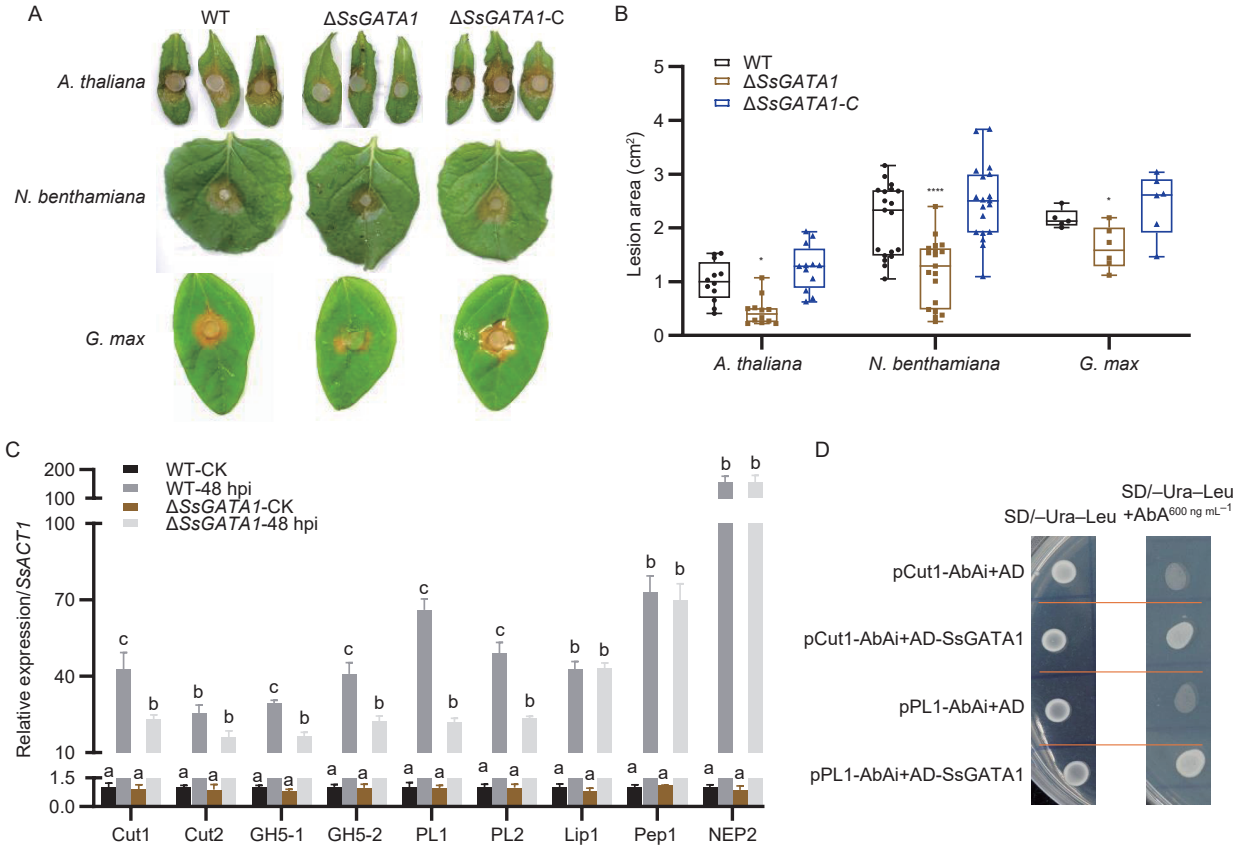


Fig. 3 SsGATA1 activates the transcription of hydrolase genes to contribute to pathogenicity. A and B, deletion of *SsGATA1* reduced pathogenicity. Disease symptoms were photographed (A), and lesion area was calculated using ImageJ (B). All individual data points are overlaid. Data were analyzed by two-way ANOVA with Sidak's multiple comparisons test compared to WT (*, $P<0.05$; ****, $P<0.0001$). C–E, SsGATA1 activates the expression of hydrolase genes during soybean (C), *Arabidopsis thaliana* (D), or *Nicotiana benthamiana* (E) infection. CK, uninoculated. Data represent the mean±SD ($n=3$). Data were analyzed by two-way ANOVA with Sidak's multiple comparisons test, and data points with different letters indicate significant differences at $P<0.05$. F, SsGATA1 binds to the promoters of *SsCut1* and *SsPL1* in Y1H assays.

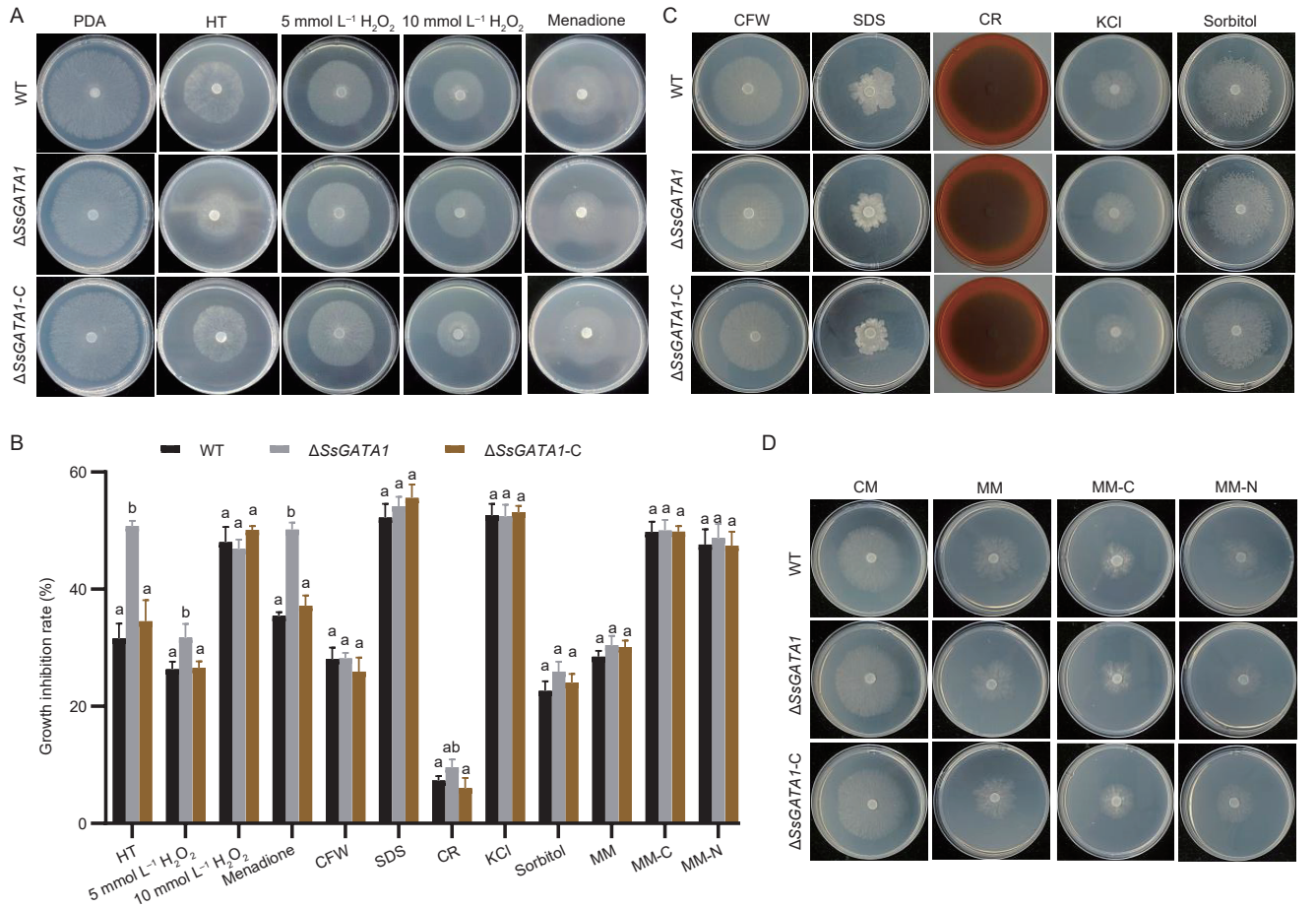


Fig. 4 SsGATA1 regulates the tolerance to high temperature (HT) stress and oxidative stress. A, SsGATA1 regulates the tolerance of *Sclerotinia sclerotiorum* to high temperature and oxidative stress. Colony morphology of the WT, Δ*SsGATA1* mutant, and Δ*SsGATA1*-C on PDA plates under untreated PDA, high temperature (HT) stress, or supplemented with 5 mmol L^{-1} H_2O_2, 10 mmol L^{-1} H_2O_2, and 30 μmol L^{-1} menadione. For HT treatment, strains were cultured on PDA plates at 25°C for 12 h, then shifted to 30°C for 24 h. B, the growth inhibition ratio was calculated. Colony diameters were measured at 36 h. The growth inhibition ratio=(Colony diameter on untreated PDA−Colony diameter on treated PDA)/(Colony diameter on untreated PDA×100%) or (Colony diameter on CM−Colony diameter on nutrient-limited medium)/(Colony diameter on CM×100%). Data represent the mean±SD (n=3). Data were analyzed by two-way ANOVA with Sidak's multiple comparisons test, and data points with different letters indicate significant differences at P<0.05. C, SsGATA1 has no effect on cell wall integrity or tolerance to hyperosmotic stress. PDA plates contained reagents that disrupt cell wall integrity: 500 μg mL^{-1} CR, 10 μg mL^{-1} CFW, and 0.01% SDS, and those that induce hyperosmotic stress: 1 mol L^{-1} KCl and 1 mol L^{-1} sorbitol. D, SsGATA1 has no effect on tolerance to nutritional stress. Colony morphology of the WT, Δ*SsGATA1* mutant, and Δ*SsGATA1*-C on CM, MM, MM-C, and MM-N plates.

genes and positively regulating their transcriptional activation upon fungicide exposure.

3.6. SsGATA1 participates in detoxification of xenobiotics

Given that SsGATA1 regulates the transcription of drug efflux pump genes, we speculated that SsGATA1 may also play a role in the efflux of antifungal xenobiotics produced by plants. As expected, compared with the WT and Δ*SsGATA1*-C, the Δ*SsGATA1* mutant was significantly more sensitive to 2PE-ITC, quercetin, or kaempferol, which inhibit the growth of *S. sclerotiorum* (Fig. 6-A and B), indicating that SsGATA1 regulates resistance to xenobiotics.

It has been reported that *S. sclerotiorum* can degrade toxic ITCs by producing ITCase to reduce ITC toxicity in planta and *in vitro*. We measured the effect of SsGATA1 on *SsSaxA* expression after 1 h of 2PE-ITC treatment and 24 h of inoculation on *A. thaliana*. The results showed that 2PE-ITC treatment or inoculation significantly induced *SsSaxA* expression, but this induction was significantly weakened in the *SsGATA1* knockout mutant (Fig. 6-C). Transcription factor-promoter binding assays showed that SsGATA1 can directly bind to the *SsSaxA* promoter (Fig. 6-D). These results indicate that SsGATA1 is involved in detoxification of antifungal xenobiotics produced by plants, at least partly through transcriptional activation of *SsSaxA*. In addition, given that ITC detoxification also contributes to *S. sclerotiorum* infection, SsGATA1 may also contribute to pathogenicity by activating *SsSaxA* expression.

4. Discussion

GATA transcription factors are reported to be widely involved in the morphogenesis, metabolism, and virulence in fungi (Scazzocchio 2000; Basse and Farfsing 2006). Here,

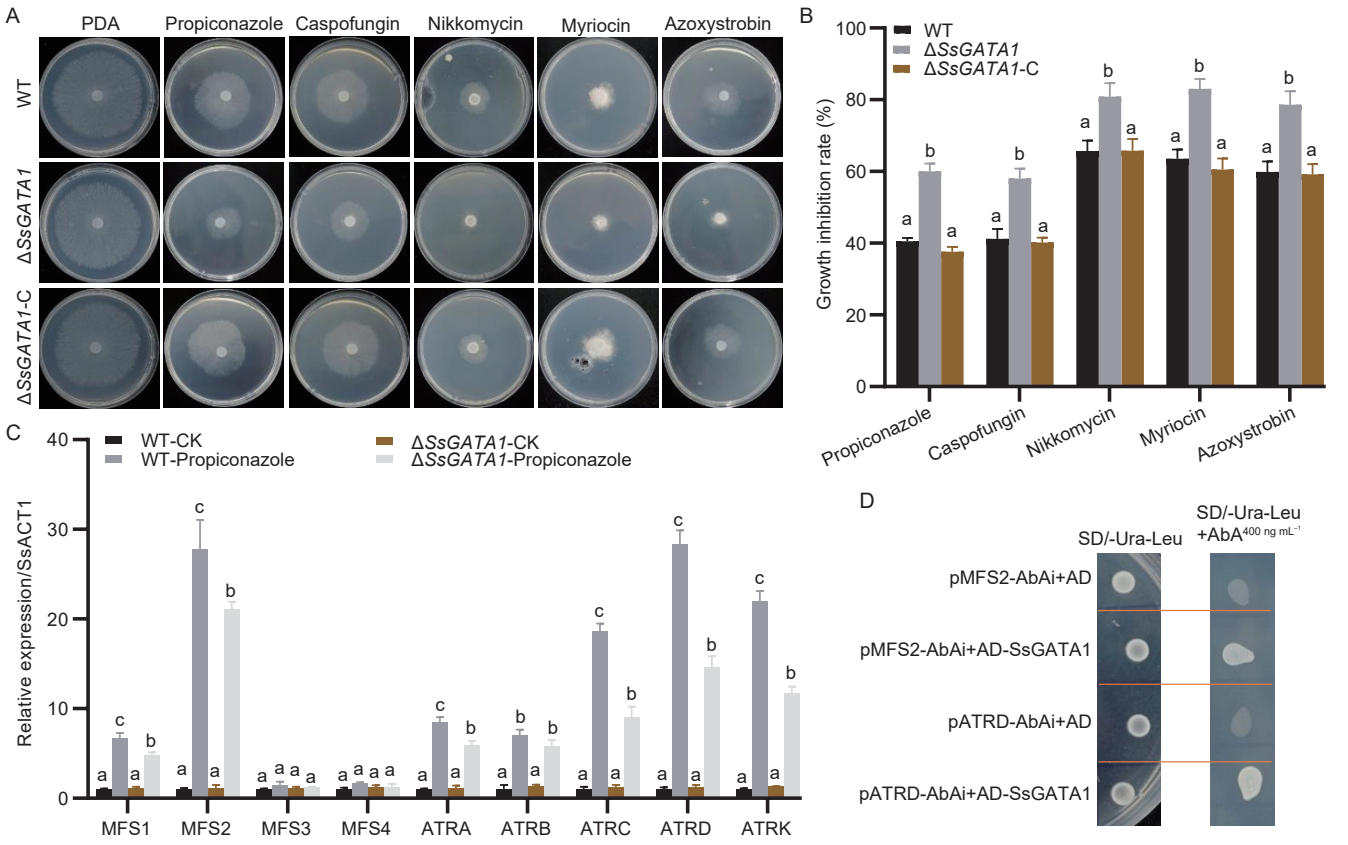


Fig. 5 SsGATA1 activates the transcription of *ABC* and *MFS* transporters to contribute to multiple drug resistance (MDR). A and B, colony morphology (A) and growth inhibition ratios (B) of the WT, Δ*SsGATA1* mutant, and Δ*SsGATA1*-C on PDA plates with 0.05 μg mL^{-1} propiconazole, 0.2 μg mL^{-1} caspofungin, 1 μg mL^{-1} nikkomycin Z, 1 μg mL^{-1} myriocin, and 0.5 ppm azoxystrobin for 36 h. The growth inhibition ratio=(Colony diameter on untreated PDA−Colony diameter on treated PDA)/(Colony diameter on untreated PDA×100%). C, SsGATA1 activates the expression of *ABC* and *MFS* transporters after treatment with propiconazole. CK, strains were cultured in untreated PDB; for propiconazole treatment, strains were cultured in PDB supplemented with 0.05 μg mL^{-1} propiconazole for 1 h. D, SsGATA1 binds to the promoters of *SsMFS2* and *SsATRD* in Y1H assays. For B and C, data represent the mean±SD (n=3). Data were analyzed by two-way ANOVA with Sidak's multiple comparisons test, and data points with different letters indicate significant differences at P<0.05.

we analyze the functions of a class of undescribed GATA transcription factors. Interestingly, unlike other GATA transcription factors that have been reported to regulate growth and development in *S. sclerotiorum*, this SsGATA1 transcription factor with GATA box binding ability does not affect growth and development but plays key roles in stress, pathogenicity, and MDR. In mechanism (Fig. 6-E), SsGATA1 positively regulates the transcriptional activation of genes encoding drug efflux pumps and xenobiotic detoxification enzymes such as *SsSaxA* in response to antifungal compounds. The contribution to pathogenicity is mainly due to the transcriptional up-regulation of genes encoding hydrolases and xenobiotic detoxification enzymes through SsGATA1 during infection.

The use of fungicides is the main measure to control most plant fungal diseases. However, this is not a once-and-for-all story. On the one hand, pathogenic fungi have the ability to survive in the presence of drug concentrations lower than the MIC (Berman and Krysan 2020), and they can also gain resistance to all major fungicides, including azoles, with the selection pressure caused by frequent use of fungicides, which leads to excessive use of fungicides and possible control failure (Fisher *et al.* 2018; Osset-Trénor *et al.* 2023). Here, we found that *S. sclerotiorum* can still grow well at several specified fungicide concentrations, but the growth at the same concentration is significantly worse after *SsGATA1* is knocked out, indicating that *S. sclerotiorum* has a certain degree of tolerance to these fungicides, of which SsGATA1 has contributed at least part. The MDR to various fungicides often puzzles agricultural producers; it is urgent to understand the MDR mechanism and the coping strategies in the face of plants or artificially applied antifungal compounds of pathogenic fungi, which is essential for the development of antifungal drugs and the exploration of alternative treatment schemes to overcome drug resistance (Osset-Trénor *et al.* 2023). A major limitation of the efficacy of fungicides is that these drugs will be quickly detoxified by pathogenic fungi through plasma membrane export. In particular, the efflux of exogenous compounds mediated by ABC and MFS drug efflux pumps plays a core role in the MDR of pathogenic fungi (Sipos and Kuchler 2006; de Waard *et al.* 2006; Gulshan and Moye-Rowley 2007). In this study, the transcription of two MFS transporters (*SsMFS1*/*2*) and five ABC transporters (*SsATRA*/*B*/*C*/*D*/*K*) of *S. sclerotiorum* was rapidly up-

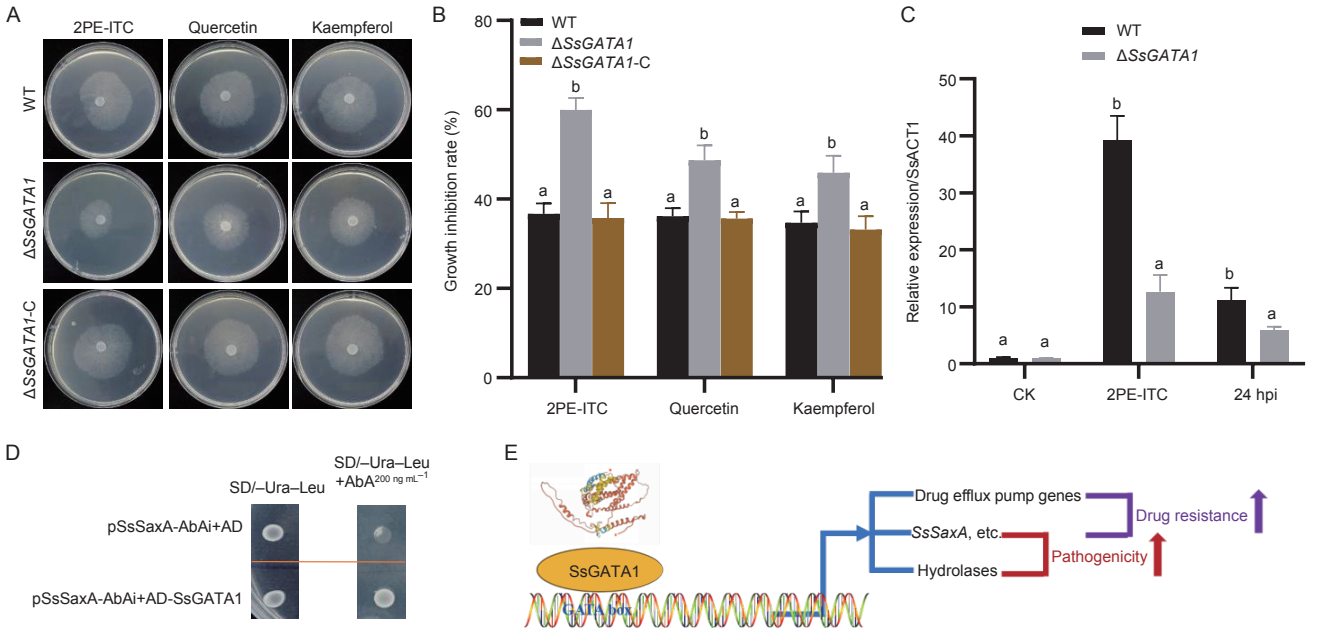


Fig. 6 SsGATA1 participates in detoxification of xenobiotics and activates the transcription of *SsSaxA*. A and B, colony morphology (A) and growth inhibition ratios (B) of the WT, Δ*SsGATA1* mutant, and Δ*SsGATA1*-C on PDA plates with 40 μmol L^{-1} 2PE-ITC, 0.02 mg mL^{-1} quercetin, and 0.02 mg mL^{-1} kaempferol for 36 h. The growth inhibition ratio=(Colony diameter on untreated PDA−Colony diameter on treated PDA)/(Colony diameter on untreated PDA×100%). C, SsGATA1 activates the expression of *SsSaxA* after treatment with 2PE-ITC or inoculation. CK, strains were cultured in untreated PDB; for 2PE-ITC treatment, strains were cultured in PDB supplemented with 40 μmol L^{-1} 2PE-ITC for 1 h; for inoculation, strains were inoculated on *Arabidopsis thaliana* for 24 h. D, SsGATA1 binds to the promoter of *SsSaxA* in Y1H assays. E, a proposed functional model for GATA transcription factor SsGATA1 in *Sclerotinia sclerotiorum*. As a GATA-type transcription factor, SsGATA1 binds to the GATA box motif in target gene promoters to activate transcription, including activating drug efflux pump genes and *SsSaxA*, etc., to promote drug resistance, and activating hydrolase genes and *SsSaxA*, etc., to contribute to pathogenicity. For B and C, data represent the mean±SD (n=3). Data were analyzed by two-way ANOVA with Sidak's multiple comparisons test, and data points with different letters indicate significant differences at P<0.05.

regulated after 1 h of propiconazole treatment, suggesting that these drug efflux pumps may play an important role in efflux of fungicides such as propiconazole. Importantly, the strains with SsGATA1 knocked out activated less expression of these drug efflux pumps under the same propiconazole stimulation, and SsGATA1 can directly bind to their promoters, suggesting that SsGATA1, as their transcriptional activator, positively regulates MDR by increasing the expression of these drug efflux pumps.

At present, reports on the transcriptional activators of drug efflux pumps in fungi are limited to the transcription factors of $Zn(II)_2Cys_6$, such as MRR1/2, TAC1, and PDR1/3 (Vermitsky and Edlind 2004; MacPherson *et al.* 2006; Thakur *et al.* 2008; Kretschmer *et al.* 2009; Lohberger *et al.* 2014; Rybak *et al.* 2020; Osset-Trénor *et al.* 2023). The common MDR strains in yeast are due to mutation of PDR1/3, which leads to over-activation of the transcription of downstream drug efflux pumps, and the synergistic effect of the transcription factors can help the overall MDR to deal with different xenobiotic compounds (Vanacloig-Pedros *et al.* 2019). In mechanism, the transcription factor PDR1/3 can bind to the pleiotropic drug resistance elements of the promoters of various drug efflux pump genes, while the regulatory domains XBD (xenobiotic binding domains) of PDR1/3 can bind to lower concentrations of ketoconazole to increase their transcriptional activity, thus activating transcription (Gao *et al.* 2004; Thakur *et al.* 2008). When the XBD part of PDR1/3 is deleted or mutated, it may lead to the generation of constitutive activity, which leads to overexpression of drug efflux pump genes and an MDR phenotype (Carvajal *et al.* 1997). In *C. albicans*, Tac1 and Mrr1 are transcription factors that are responsible for activating *Cdr1*, *Cdr2*, or *Mdr1* transporters, and point mutations or deletion mutations of Tac1 or Mrr1 enhance the transactivation capacity in the presence of additional transcriptional co-activators to confer MDR (Liu and Myers 2017a, b). Similarly, Mrr1 also regulates the transcription of *BcatrB*, and acquired mutation of Mrr1 can cause overexpression of *BcatrB* and lead to an MDR phenotype in *B. cinerea* (Kretschmer *et al.* 2009; Copier *et al.* 2024). Different from the previous $Zn(II)_2Cys_6$ transcription factors, a new transcriptional activator of drug efflux pumps, GATA transcription factor SsGATA1, was identified in this study, which showed that SsGATA1 could directly bind to the promoters of several drug efflux pump genes to activate their transcription and contribute to MDR. To the best of our knowledge, this is the first report on a transcriptional activator of drug efflux pumps found in *S. sclerotiorum*. In the absence of drugs, SsGATA1 does not affect the transcription level of drug efflux pump genes; therefore, it will be an interesting project to further study how SsGATA1 senses the presence of drugs to manipulate the transcriptional activation of drug efflux pumps, which will help to reveal the molecular mechanism of antifungal drug resistance of *S. sclerotiorum* and deepen our understanding of transcriptional regulation of

MDR in plant pathogenic fungi.

Interestingly, transcriptional machineries that negatively regulate fungicide resistance were also found in pathogenic fungi (Meng *et al.* 2023). The velvet family proteins may reduce the resistance of rice blast fungus to isoprothiolane by expressing secondary metabolism-related genes or *CYP450* genes, which is manifested by the downregulation of these downstream genes and the improvement of resistance to isoprothiolane after mutation of the velvet family proteins. We speculate that these transcriptional machineries themselves are key factors for fungal growth and sporulation, rather than specialized proteins for manipulating fungicide resistance, because isoprothiolane resistance is at the expense of reduced mycelial growth, sporulation, oxidative stress tolerance, and resistance to fludioxonil and iprodione. Under the continuous selection pressure of isoprothiolane, the down-regulation of secondary metabolism-related genes or *CYP450* genes caused by mutation of the velvet family proteins may reduce the modification and activation of isoprothiolane, thus reducing its toxicity. Whether SsGATA1 similarly changes the modification and activation of fungicides remains to be further explored, although we tend to think that this is unlikely because SsGATA1 has the characteristics of enhancing resistance to broad-spectrum fungicides.

As a typical necrotrophic plant pathogen with a widespread host range, *S. sclerotiorum* can secrete a large number of hydrolases to degrade plant cell components during infection, such as cutinase to break the outer barrier of plants and various cell wall-degrading enzymes to degrade plant cell walls to promote infection and provide nutrition for growth (Bolton *et al.* 2006). Many transcriptome reports about the interaction between *S. sclerotiorum* and its host show that these hydrolases are highly up-regulated during infection, and several reports describe their functions (Seifbarghi *et al.* 2017; Westrick *et al.* 2019; Gong *et al.* 2022; Wei *et al.* 2022). However, it is still a mystery which transcription factors regulate the timely up-regulation of these hydrolases. Here, we found that SsGATA1 can bind to the promoters of several hydrolase genes to activate their transcription to promote the maceration ability of *S. sclerotiorum*; therefore, we believe that SsGATA1 is the transcriptional activator of hydrolase genes. Interestingly, SsGATA1 does not affect the expression level of hydrolase genes when uninfected, which implies that SsGATA1 can also activate the transcription of target genes in response to the presence of hydrolase substrates or the progress of infection. There are at least three reasons for the obvious decline in pathogenicity after deleting *SsGATA1*. First, the ability of mutants to express hydrolases decreased. Second, SsGATA1 may resist the oxidative stress imposed by plants during infection by enhancing its antioxidant capacity. The evidence is that SsGATA1 also mediates tolerance to oxidative stress, which may be through transcription of ABC transporters, as previously reported (Sun *et al.* 2006), and the antioxidant capacity of *S. sclerotiorum* is related to pathogenicity (Xiao *et al.* 2024). Third, SsGATA1 also promotes infection by reducing the antifungal compounds produced by plants during infection. We have confirmed that SsGATA1 can directly bind to the promoter of *SsSaxA* to activate its transcription during infection and participate in detoxification of ITC with anti-*S. sclerotiorum* activity. *In vitro* experiments have also proved that SsGATA1 can activate the transcription of *SsSaxA* after responding to ITC for 1 h. In addition, SsGATA1 may also regulate tolerance to several antifungal xenobiotics produced by plants by mediating the transcription of ABC and MFS transporters. The ABC and MFS transporters have been reported in several filamentous fungi to participate in the pathogenic process through efflux of antifungal xenobiotics produced by plants (Sun *et al.* 2006; de Waard *et al.* 2006; Stefanato *et al.* 2009; Wu *et al.* 2024).

5. Conclusion

In summary, we functionally characterized the GATA transcription factor SsGATA1 in *S. sclerotiorum*, which is a novel transcription activator of drug efflux pumps. SsGATA1 can mediate MDR by activating the transcription of drug efflux pump genes and also act as the transcription activator of hydrolase genes and *SsSaxA*, respectively, to regulate the hydrolysis of plant tissues and detoxification of xenobiotics produced by plants during infection, jointly playing a pathogenic role. Identification of important pathogenic factors in pathogens and their application as drug targets has been applied in the control of plant diseases; therefore, new fungicides or drugs can be developed based on SsGATA1 as a drug target. In addition, considering the role of SsGATA1 in MDR, inhibiting its function is expected to improve the efficacy of fungicides. For example, combination therapy using drugs targeting SsGATA1 and common fungicides will reduce the dosage of fungicides.

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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

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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