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The host and pathogen *myo*-inositol-1-phosphate synthases are required for *Rhizoctonia solani* AG1-IA infection in tomato

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Abstract

The *myo*-inositol-1-phosphate synthase (MIPS) catalyses the biosynthesis of *myo*-inositol, an important sugar that regulates various physiological and biochemical processes in plants. Here, we provide evidence that host (*SlMIPS1*) and pathogen (*Rs_MIPS*) *myo*-inositol-1-phosphate synthase (*MIPS*) genes are required for successful infection of *Rhizoctonia solani*, a devastating necrotrophic fungal pathogen, in tomato. Silencing of either *SlMIPS1* or *Rs_MIPS* prevented disease, whereas an exogenous spray of *myo*-inositol enhanced disease severity. *SlMIPS1* was upregulated upon *R. solani* infection, and potentially promoted source-to-sink transition, induced *SWEET* gene expression, and facilitated sugar availability in the infected tissues. In addition, salicylic acid (SA)-jasmonic acid homeostasis was altered and SA-mediated defence was suppressed; therefore, disease was promoted. On the other hand, silencing of *SlMIPS1* limited sugar availability and induced SA-mediated defence to prevent *R. solani* infection. Virus-induced gene silencing of *NPR1*, a key gene in SA signalling, rendered *SlMIPS1*-silenced tomato lines susceptible to infection. These analyses suggest that induction of SA-mediated defence imparts disease tolerance in *SlMIPS1*-silenced tomato lines. In addition, we present evidence that *SlMIPS1* and SA negatively regulate each other to modulate the defence response. SA treatment reduced *SlMIPS1* expression and *myo*-inositol content in tomato, whereas *myo*-inositol treatment prevented SA-mediated defence. We emphasize that downregulation of host/pathogen MIPS can be an important strategy for controlling diseases caused by *R. solani* in agriculturally important crops.

KEYWORDS
necrotrophs, phytohormones, SA-mediated defence, sheath blight disease, sugar signalling, susceptibility genes

1 | INTRODUCTION

Plants and pathogens have been co-evolving to establish dominance over each other. Plants use various approaches to fortify their defence, whereas pathogens adopt diverse strategies to circumvent host defence responses (Yuan et al., 2021). Pathogens also modulate important host cellular machinery (genes/pathways), commonly referred to as susceptibility (*S*) genes (Langner et al., 2018; Zaidi et al., 2018) that are important for the growth and development of plants (Engelhardt et al., 2018; Gorshkov & Tsers, 2022; Nunes da Silva et al., 2022; Rani & Jha, 2020). The loss-of-function mutation or silencing of the *S*-gene compromises the pathogen's ability to colonize host plants and cause disease (Kieu et al., 2021; Lapin & Van den Ackerveken, 2013; van Schie & Takken, 2014). One of the best-characterized examples of the *S*-gene is the mildew resistance gene locus O (*Mlo*) in barley. The naturally occurring mutant alleles of *Mlo* provide broad-spectrum resistance against various strains of powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) in barley. Orthologues of *Mlo* promote disease susceptibility in plants, including *Arabidopsis thaliana*, pea, tomato, pepper, wheat, and strawberry (Acevedo-Garcia et al., 2014). *S*-genes against various phytopathogens have been identified, for example, *OsRACs* (*Magnaporthe oryzae*), *OsWRKYs* (van Schie & Takken, 2014), *OsSWEET11* (*Xanthomonas oryzae*) (Xu et al., 2019), and Downy mildew resistant 1 (*AtDMR1*) (*Hyaloperonospora arabidopsidis*) (Sun et al., 2016). In recent years, modulation of *S*-genes has emerged as an important strategy to impart disease tolerance, particularly when suitable resistance (*R*)-genes are not available (Garcia-Ruiz et al., 2021; Zafar et al., 2020; Zaidi et al., 2018).

Rhizoctonia solani strains belonging to different anastomosis groups pose serious threats to agriculture (Francis et al., 2023; Molla et al., 2020; Zhao et al., 2021). Due to a lack of resistant resources, it is difficult to control *R. solani* infection in crop plants, including rice and tomato. Genome, transcriptome, proteome, and metabolome-based studies have suggested that the pathogen adopts diverse strategies to colonize a wide range of plant species (Ball et al., 2020; Fordyce et al., 2018; Francis et al., 2023; Ghosh et al., 2014; Peyraud et al., 2017). *R. solani* infection causes major metabolic reprogramming, including alteration of respiration, secondary metabolism, carbon metabolism, and hormonal signalling in host plants (Acharya et al., 2022; Anderson et al., 2017; Ghosh et al., 2017; Kidd et al., 2021; Kouzai et al., 2018; Yang et al., 2022; Yuan et al., 2020). However, the importance of such an alteration in promoting *R. solani* infection remains to be emphatically demonstrated. Previously, we have identified certain rice genes, including *NUOR* (alternative NADH:Ubiquinone Oxidoreductase, a component of the mitochondrial electron transport chain) and *MIPS* (*myo*-inositol-1-phosphate synthase), that are differentially expressed during *R. solani* AG1-IA infection (Ghosh et al., 2017). The silencing of host *NUOR* compromised the pathogen to infect rice as well as tomato (Kant et al., 2019). MIPS is widely conserved in diverse organisms, including plant, bacterial, and fungal species (Basak et al., 2018; Majumder et al., 2003). It catalyses the rate-limiting step in the biosynthesis of *myo*-inositol (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol) from glucose 6-phosphate (Loewus & Loewus, 1983; Loewus & Murthy, 2000). In plants, *myo*-inositol is involved in important physiological/biochemical processes, including membrane biogenesis, programmed cell death (Tan et al., 2013), growth regulation, hormonal regulation, and stress signalling (Saxena et al., 2013; Tan et al., 2013). The overexpression of *MIPS* provides tolerance against heat stress in wheat (Khurana et al., 2017), and salt and drought stress in rice (Das-Chatterjee et al., 2006), as well as in sweet potato (Das-Chatterjee et al., 2006; Joshi et al., 2013; Zhai et al., 2016). *myo*-Inositol is also required to promote mutualistic interactions between plants and beneficial rhizobacteria (Vílchez et al., 2020).

In the present study, we provide evidence that *R. solani* upregulates its endogenous (*Rs_MIPS*; *R. solani myo-inositol-1-phosphate synthase*) as well as host (*SlMIPS1; Solanum lycopersicum myo-inositol-1-phosphate synthase 1*) *MIPS* genes to promote infection in tomato. The silencing of *SlMIPS1* or *Rs_MIPS* prevents *R. solani* infection, whereas exogenous *myo*-inositol treatment enhances disease severity. Moreover, heterologous overexpression of *SlMIPS1* renders *atmips1* mutant *Arabidopsis thaliana* plants susceptible to infection. We emphasize that upregulation of *SlMIPS1* potentially assists in nutrient acquisition, prevents SA (salicylic acid)-mediated defence, and enhances necrotrophy during *R. solani* infection in tomato. On the other hand, silencing of *SlMIPS1* induces SA-mediated defence, alters sugar signalling, and imparts disease tolerance. Overall, our data suggest that *SlMIPS1* and SA negatively regulate each other to modulate *R. solani* infection in tomato.

2 | RESULTS

2.1 | Host *myo*-inositol-1-phosphate synthase is required for successful pathogenesis of *R. solani* in tomato

A previous study revealed that the *Oryza sativa myo*-inositol-1-phosphate synthase gene (*OsMIPS*) is upregulated upon *R. solani* infection in rice (Ghosh et al., 2017). There are two paralogues of *OsMIPS* [*OsMIPS1* (LOC_Os03g09250) and *OsMIPS2* (LOC_Os10g22450)] in rice (Figure S1a). Analysis using the Gene Investigator tool reflected that *OsMIPS1* is expressed in different rice tissues (Figure S2a), whereas *OsMIPS2* is mainly expressed in the reproductive tissues (Figure S2b). Reverse transcription-quantitative PCR (RT-qPCR) analysis revealed that *OsMIPS1* was highly upregulated in the *R. solani*-infected rice (cv. PB1) at 3 days post-inoculation (dpi), whereas *OsMIPS2* showed only limited upregulation compared to uninfected samples (Figure S2c). Therefore, we selected *OsMIPS1* to investigate its role during *R. solani* infection. An attempt was made to generate *OsMIPS1*-silenced stable RNAi rice lines, and only two positive lines with reduced *myo*-inositol content were obtained (Figure S3a). However, both lines showed very poor growth (Figure S3b), and they mostly

produced chaffy seeds (Figure S3c). This correlates with published reports that silencing of *OsMIPS1* has a detrimental effect on the growth and development of rice (Feng & Yoshida, 2004; Kuwano et al., 2009).

Considering that *R. solani* can infect tomato, an economically important horticultural crop that is amicable for functional studies (Chandan, Kumar, Kabyashree, et al., 2023; Ghosh et al., 2021; Kant et al., 2019; Swain et al., 2019), we attempted to identify OsMIPS1 homologues in tomato. BLAST searches in the SOLGenomics database identified five *Solanum lycopersicum* MIPSs (SlMIPSs) paralogues (Figure S1b), and phylogenetic analysis reflected that they were distinct from those of rice and *A. thaliana* (Figure S4). SlMIPS1 (Solyc04g054740) and SlMIPS4 (Solyc05g051850) showed high sequence homology with that of OsMIPS1/AtMIPS1. As *SlMIPS1* was highly upregulated at 3 and 5 dpi of *R. solani* infection in tomato (cv. Pusa Ruby) (Figure S5a), we selected it for further analysis. The *SlMIPS1*pro:GUS reporter assay reflected that *SlMIPS1* expression was induced under infected conditions (Figure S6a,b). To investigate further, we obtained stable RNAi tomato lines and observed a direct correlation between the downregulation of *SlMIPS1* (Figure S7a) with reduced *myo*-inositol content (Figure S7b) and enhanced disease tolerance (Figure S7c,d) at T_2 generation. Two of the silenced lines (L2 and L7) with a significant reduction in *myo*-inositol content and disease symptoms were further analysed at T_3 generation. Both of these lines exhibited a normal vegetative growth phenotype, although they produced smaller fruits compared to WT (wild-type)/EV (empty vector transformed) control plants (Figure S8a–c). RT-qPCR analysis reflected that *SlMIPS1* was downregulated, while the expression of other *SlMIPSs* (*SlMIPS2–5*) remained unaltered in the silenced lines (Figure S5b). Besides *SlMIPS1* downregulation (Figure 1a), *myo*-inositol content was significantly reduced in the silenced lines upon *R. solani* infection (Figure 1b).

Most notably, *SlMIPS1*-silenced lines were tolerant to *R. solani* infection, as they had significantly reduced necrotic symptoms as well as reactive oxygen species (ROS) accumulation (Figure 1c), lesion area (Figure 1d), and pathogen load (Figure 1e), compared to wild-type (WT)/empty vector (EV) plants. The exogenous spray of *myo*-inositol (100 mM) rendered *SlMIPS1*-silenced plants susceptible

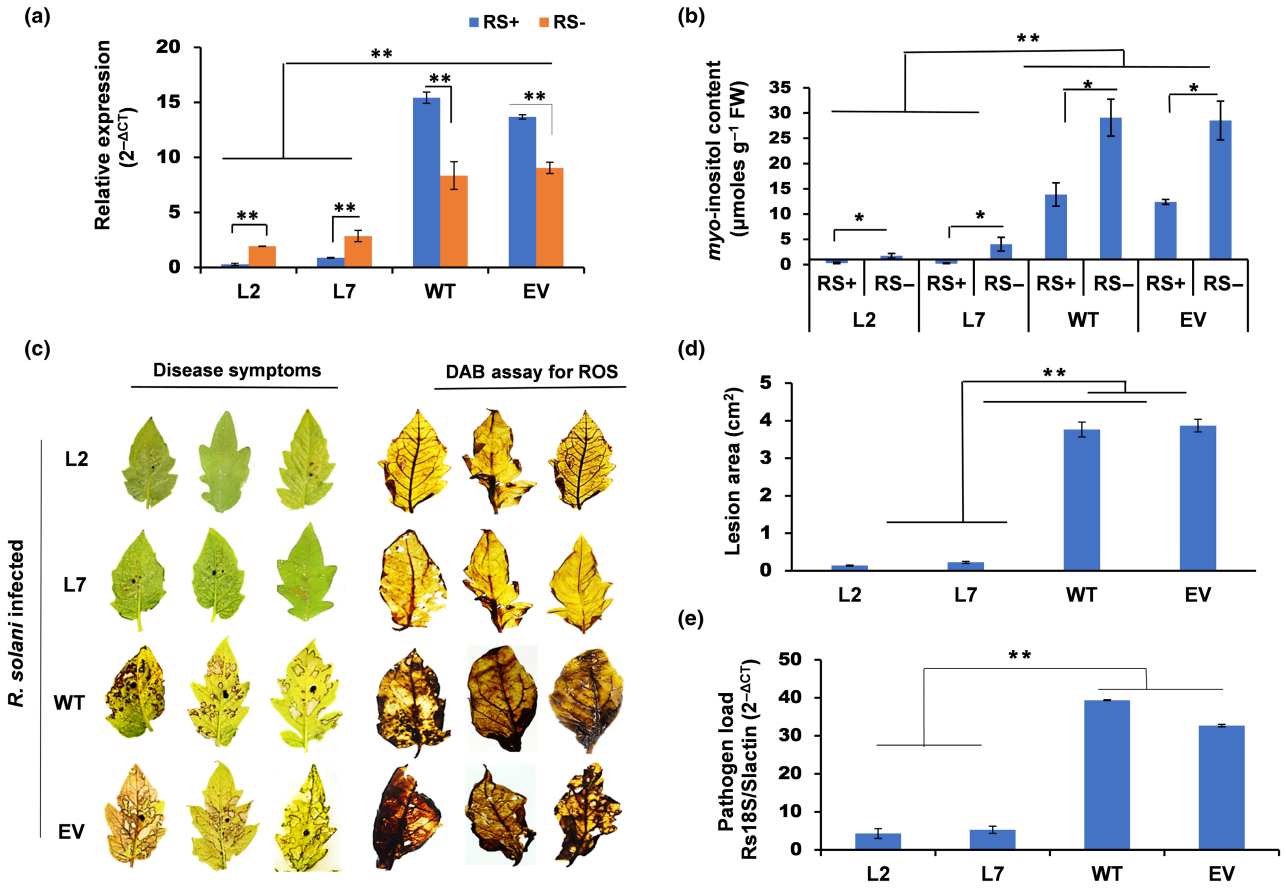

FIGURE 1 Silencing of *SlMIPS1* prevents *Rhizoctonia solani* infection in tomato. (a) Reverse transcription-quantitative PCR-based quantification of *SlMIPS1* expression (gene expression was normalized using tomato *actin* as an endogenous control), and (b) gas chromatography-mass spectrometry-based quantification of *myo*-inositol content in *R. solani*-infected (RS+, 3 days post-inoculation [dpi]) and uninfected (RS−) tomato leaves. (c) Disease symptoms, and reactive oxygen species (ROS) accumulation (3,3′-diaminobenzidine [DAB] staining), (d) lesion area, and (e) pathogen load (estimated as an abundance of 18S rRNA of *R. solani* using tomato *actin* as an endogenous control) in the infected leaves, at 3 dpi. The graph shows the mean values ± *SE* of three biological replicates. WT, wild type; EV, empty vector; L2 and L7, lines silenced for *SlMIPS1* using RNAi. Significant differences, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

to *R. solani* infection. The disease symptoms (Figure S9a), lesion area (Figure S9b), and pathogen load (Figure S9c) were significantly enhanced in *myo*-inositol-treated *SlMIPS1*-silenced plants compared to buffer-treated ones. On the other hand, sorbitol (100 mM) treatment (used as an osmolyte control) failed to promote disease in *SlMIPS1*-silenced plants (Figure S9a–c). Overall, these results emphasized that *myo*-inositol is important for *R. solani* infection in tomato, and knocking down *SlMIPS1* provides disease tolerance. In support of this, we observed that *atmips1* mutant *A. thaliana* (Hu et al., 2018, 2020) plants were tolerant to *R. solani* infection, while ectopic overexpression of *SlMIPS1* enhanced disease severity (Figure S10b), lesion area (Figure S10c), and pathogen load (Figure S10d).

2.2 | dsRNA-mediated silencing of *Rs_MIPS* compromises *R. solani* to infect plants

It was intriguing that although *SlMIPS1* expression was enhanced (Figure 1a), *myo*-inositol content was reduced in WT/EV plants under infected conditions (Figure 1b). Considering that *myo*-inositol catabolism is important for the virulence of a bacterial wilt pathogen (*Ralstonia solanacearum*) in tomato (Hamilton et al., 2021), we anticipated that *R. solani* might take up *myo*-inositol from the host to sustain its growth during the infection process. To investigate further, we tested whether *myo*-inositol could directly modulate *R. solani* growth under laboratory conditions. The data reflected that exogenous supplementation of *myo*-inositol (50 μM to 1 mM) significantly enhanced fungal growth on minimal medium (Czapek Dox agar; CDA) plates (Figure S11a,b). However, at higher concentrations (2 mM), it failed to enhance the growth. Sorbitol supplementation (osmolyte control) did not affect *R. solani* growth on CDA plates.

Considering that MIPS is conserved in fungi (Majumder et al., 2003), we attempted to identify and characterize the role of *R. solani* MIPS. BLAST searches in the rice sheath blight database (https://nipgr.ac.in/RSB/) that harbours the genomic data of *R. solani* AG1-IA strain BRS1 (our laboratory strain) (Francis et al., 2023) identified a MIPS-encoding gene (*R. solani MIPS*; *Rs_MIPS*, *Rs_10081.1*). Phylogenetic analysis reflected that Rs_MIPS is clustered with the MIPS of other basidiomycete fungi, while it forms a distinct clade with that of plants (Figure S4). RT-qPCR analysis revealed that *Rs_MIPS* was upregulated during *R. solani* infection in rice (Figure S12a) and tomato (Figure S12b). As genetic transformation of *R. solani* remains a major limitation for gene function analysis,

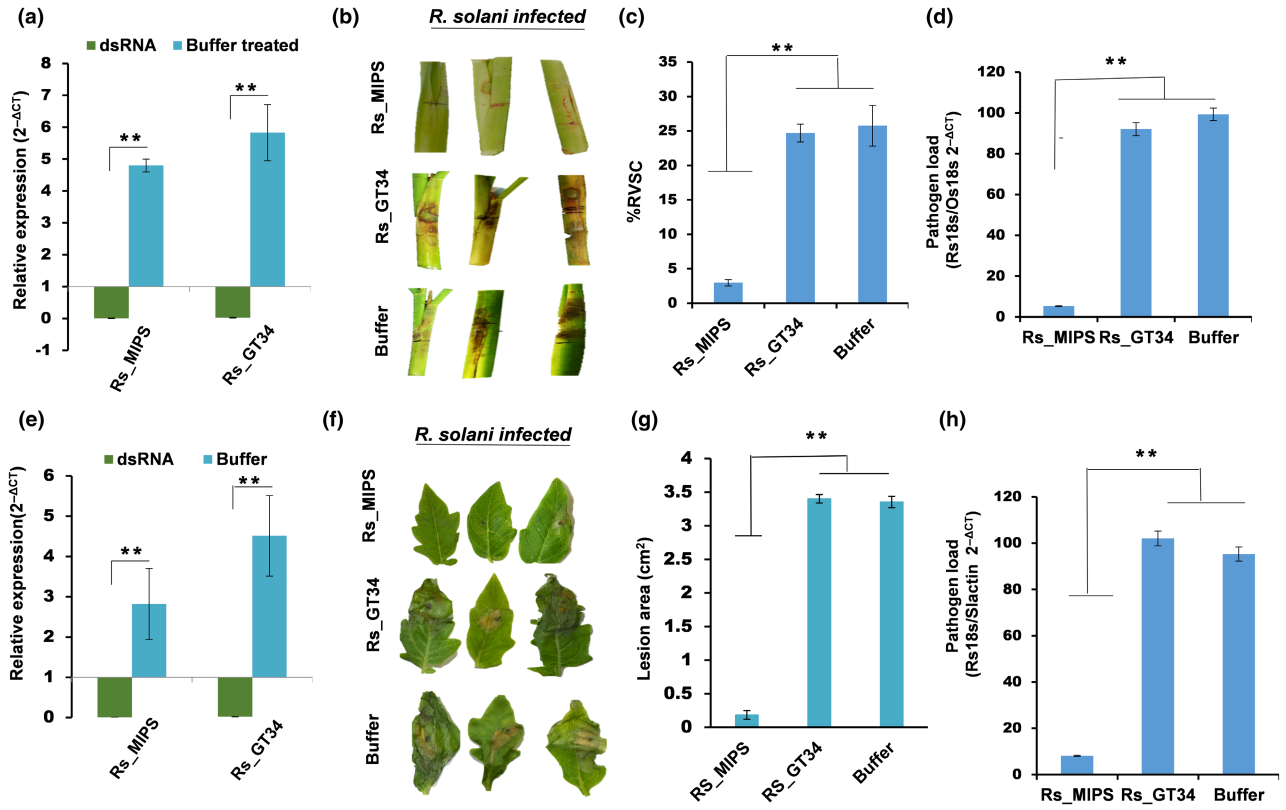

FIGURE 2 dsRNA-mediated silencing of *Rs_MIPS* compromises *Rhizoctonia solani's* ability to infect plants. (a) Reverse transcription-quantitative PCR (RT-qPCR)-based expression analysis of *Rs_MIPS* and *Rs_GT34* genes, (b) disease symptoms, (c) disease severity (% relative vertical sheath colonization [RVSC]), and (d) pathogen load in *R. solani*-infected rice, with and without dsRNA treatment, at 3 days post-inoculation (dpi). (e) RT-qPCR analysis of expression of *Rs_MIPS* and *Rs_GT34*, (f) disease symptoms, (g) lesion area, and (h) pathogen load in *R. solani*-infected tomato, with and without dsRNA treatment, at 3 dpi. The expression of the *R. solani* genes was normalized using 18S rRNA of *R. solani* as an endogenous control. Pathogen load was estimated as an abundance of 18S rRNA in *R. solani* using rice 18S rRNA/tomato *actin* as an endogenous control. The graph shows the mean values ± *SE* of three biological replicates. Significant difference, $^{**}p \leq 0.01$.

we adopted a double-stranded RNA (dsRNA)-based gene-silencing approach (Ghosh et al., 2021) to knock down *Rs_MIPS* and study its role during plant colonization (Figure 2). The *Rs_GT34* (*R. solani glycosyl transferase family 34*) gene was used as a negative control, as its silencing does not compromise *R. solani* infection in plants (Francis et al., 2023; Ghosh et al., 2021). The dsRNA treatment was efficient in knocking down the expression of target genes (*Rs_MIPS* and *Rs_GT34*) during infection in rice (Figure 2a) and tomato (Figure 2e). Moreover, compared to buffer-treated or *Rs_GT34*-silenced (control), *Rs_MIPS*-silenced *R. solani* was compromised in causing disease in rice (Figure 2b–d) and tomato (Figure 2f–h). The disease symptoms (Figure 2b,f), disease severity (represented as % RVSC [relative vertical sheath colonization] in rice, Figure 2c, and lesion area in tomato, Figure 2g) and pathogen load (Figure 2d,h) were significantly reduced in plants infected with *Rs_MIPS*-silenced *R. solani*, compared to those infected with buffer-treated or *Rs_GT34*-silenced *R. solani*.

Being a necrotrophic pathogen, *R. solani* grows in an oxidative-stress-enriched environment in the host (Das et al., 2024; Ghosh et al., 2017; Kant et al., 2019; Kumar et al., 2023; Molla et al., 2020). We observed that the growth of *Rs_MIPS*-silenced *R. solani* was compromised on CDA plates (Figure S13a,b). Notably, the growth defect was more prominent in the presence of H_2O_2 (hydrogen peroxide, as a mimic of oxidative stress), and it was restored upon supplementation with *myo*-inositol (Figure S13c,d). These results suggest that *Rs_MIPS*, as well as *myo*-inositol, play an important role in the management of oxidative stress during host colonization.

2.3 | Sugar metabolism is altered upon *R. solani* infection in tomato

Considering that *myo*-inositol plays a pivotal role in carbohydrate metabolism in plants (Loewus & Murthy, 2000) (Figure 3a), using gas chromatography-tandem mass spectrometry (GC–MS/MS), we measured sucrose, fructose, and trehalose content in different tomato lines. Under uninfected conditions, the sucrose to fructose ratio was comparable in different lines (Figure S14), while trehalose, being present in trace amounts in plants (Fichtner & Lunn, 2021), remained undetected. However, under *R. solani*-infected conditions, the sucrose to fructose ratio (Figure 3b) was significantly reduced, while trehalose content was enhanced (Figure 3c) in *SlMIPS1*-silenced lines compared to WT/EV plants. We also analysed the expression of sugar metabolism-related genes (sucrose synthase [*SUS*], lactate dehydrogenase [*LDH*], and hexokinases [*HK1*]) and transport (paralogs of Sugars Will Eventually be Exported Transporter, *SWEET11* [*11A*, *11B*, *11C*, and *11D*] and *SWEET12* [*12A* and *12C*]; Feng et al., 2015) in tomato. Amongst them, *LDH*, *SUS*, and *HK1* were significantly induced (Figure 3d), while *SWEET11* (*11A*, *11B*, *11C*, and *11D*) and *SWEET12* (*12A* and *12C*) were downregulated (Figure 3e) in *SlMIPS1*-silenced lines under infected conditions, compared to the

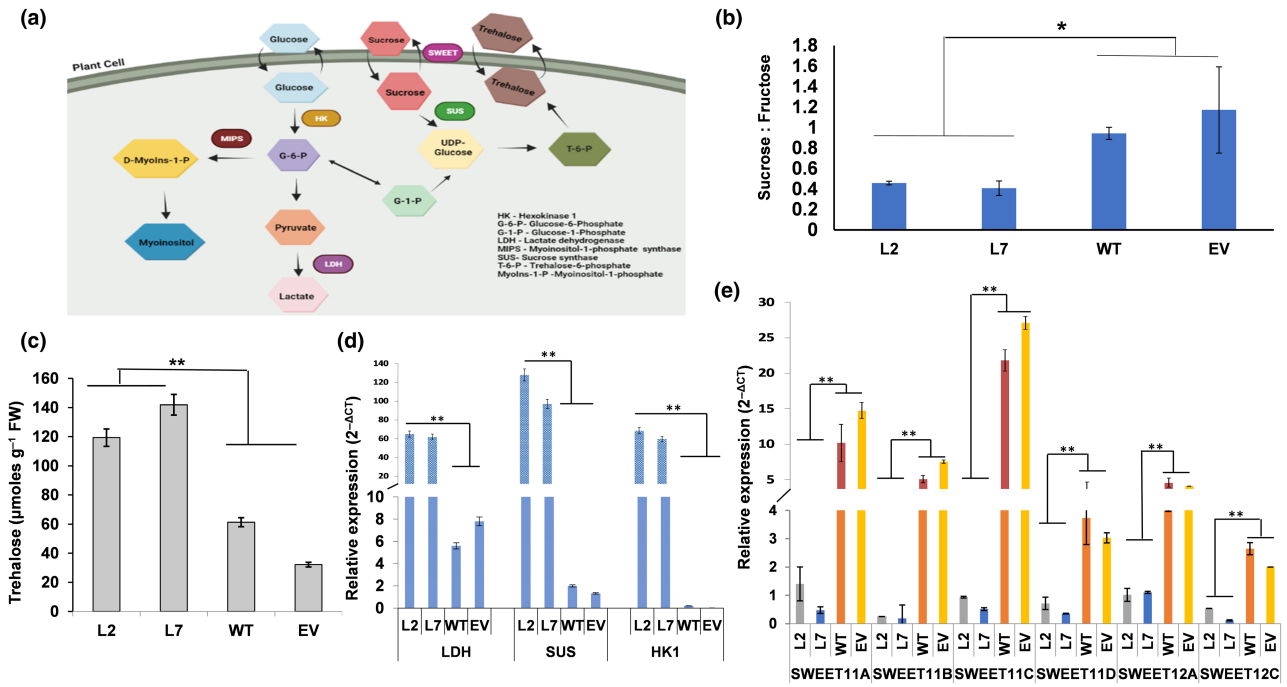

FIGURE 3 Sugar metabolic pathways are altered during *Rhizoctonia solani* infection in tomato. (a) Overview of cross-talk between sugar metabolic pathways in plants. The involvement of MIPS is highlighted. Gas chromatography-mass spectrometry-based quantification of (b) sucrose:fructose ratio and (c) trehalose content in *R. solani* infected (3 days post-inoculation [dpi]) tomato lines. (d) Reverse transcription-quantitative PCR-based expression analysis of lactate dehydrogenase (*LDH*), sucrose synthase (*SUS*), hexokinase1 (*HK1*), and (e) *SWEET* genes in the infected tomato lines, at 3 dpi. The expression of target genes was normalized using tomato *actin* as an endogenous control. The graph shows the mean values ± *SE* of three biological replicates. WT, wild type; EV, empty vector; L2 and L7, lines silenced for *SlMIPS1* using RNAi. Significant difference, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$.

WT/EV plants. Under uninfected conditions, expression of these genes was comparable in WT/EV and *SlMIPS1*-silenced tomato lines (Figure S14b,c).

2.4 | Induction of SA-mediated defence prevents *R. solani* infection in *SlMIPS1*-silenced tomato

Considering that *myo*-inositol modulates hormonal regulation in plants (Fàbregas & Fernie, 2021; Hu et al., 2020), we quantified the level of defence-related phytohormones (SA, jasmonic acid [JA], jasmonic acid isoleucine [JA-Ile], and abscisic acid [ABA]) and also analysed the expression of defence marker genes (SA, JA, ethylene [ET]-responsive genes, Table S2) in tomato. The levels of SA, JA, and ET (Figure S15a) and the expression of SA-, JA-, and ET-responsive genes (Figure S15b–d) were mostly comparable in different tomato lines under uninfected conditions. However, upon *R. solani* infection, SA was significantly enhanced (400 ng/g) and JA was reduced in *SlMIPS1*-silenced lines compared to that of WT/EV plants (Figure 4a). Moreover, SA-responsive genes including *NPR1* (Figure 4b), *PRs* (*PR1*, *PR2*, *PR4*, and *PR5*) (Figure 4c), as well as *WRKY33*, *MPK4*, and *MKP6* (Figure 4d), were significantly up-regulated in *SlMIPS1*-silenced lines under infected conditions. On the other hand, the expression of JA- (*AOS2*) and ET-responsive (*EIN3*) genes mostly remained comparable in *SlMIPS1*-silenced and WT/EV tomato lines (Figure 4b).

Considering that NPR1 plays an important role in the elaboration of SA-mediated defence (Backer et al., 2019; Boatwright & Pajerowska-Mukhtar, 2013; Wu et al., 2012), we silenced the gene in *SlMIPS1*-silenced tomato lines (L2 and L7) using the virus-induced gene-silencing (VIGS) approach (Figure 5a). Notably, necrotic symptoms (Figure 5b) and pathogen load (Figure 5c) were significantly enhanced in *NPR1*-silenced (pTRV:*NPR1*-infiltrated) plants compared to control (pTRV:0-infiltrated, empty vector) plants. Taken together, our data emphasizes that SA signalling is important for the elaboration of defence responses in *SlMIPS1*-silenced tomato lines.

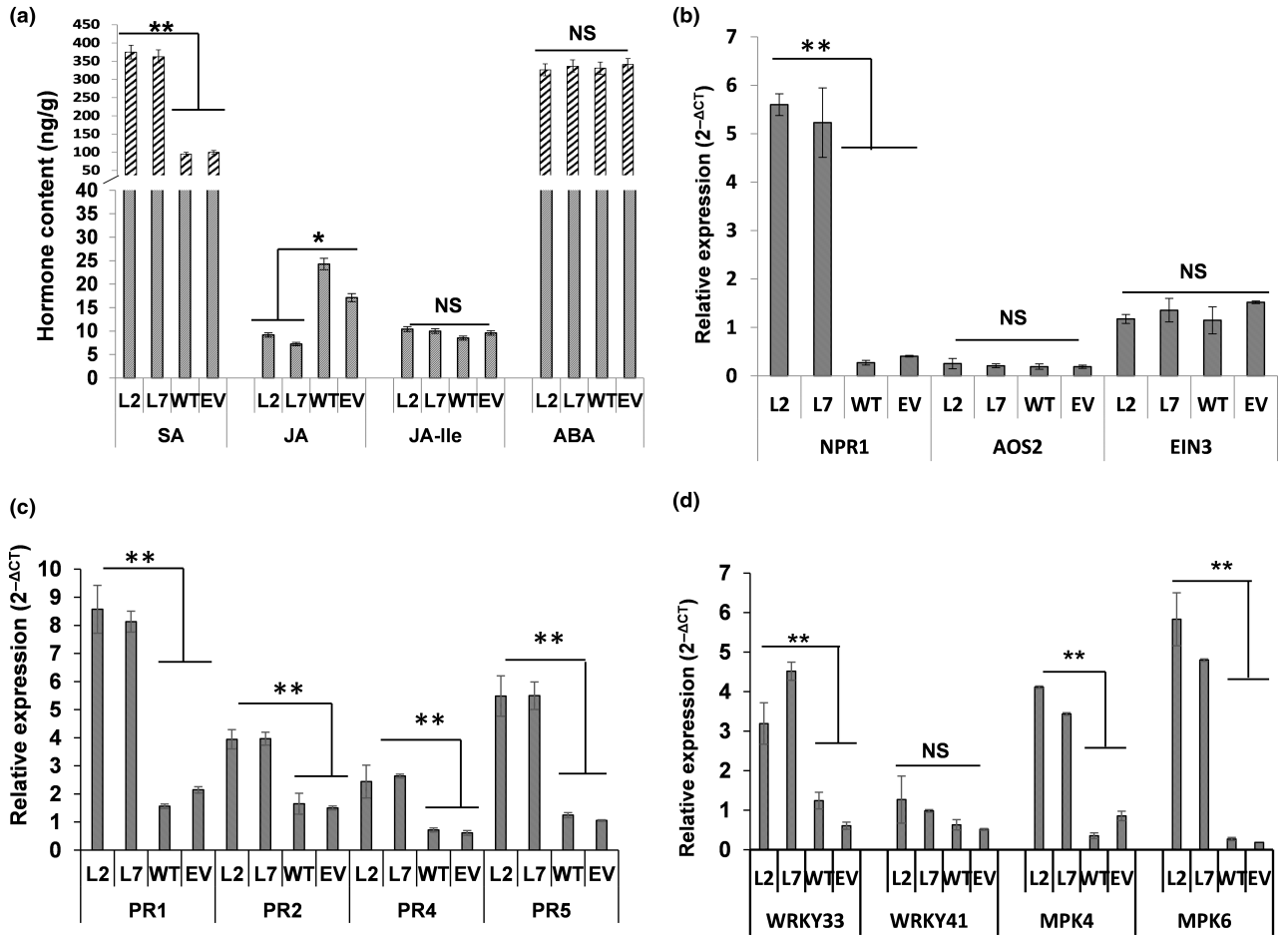

FIGURE 4 The phytohormone-mediated defence response is modulated during *Rhizoctonia solani* infection in tomato. (a) Liquid chromatography-tandem mass spectrometry-based quantification of various defence phytohormones. SA, salicylic acid; JA, jasmonic acid; JA-Ile, jasmonic acid isoleucine; ABA, abscisic acid. Reverse transcription-quantitative PCR-based expression analysis of (b) phytohormone-mediated defence genes (*NPR1*, *AOS2*, and *EIN3*), (c) pathogenesis-related genes (*PR1*, *PR2*, *PR4*, and *PR5*), and (d) *WRKY33*, *WRKY41*, *MPK4*, and *MPK6* genes in *R. solani*-infected (3 days post-inoculation) tomato lines. The gene expression was normalized using tomato *actin* as an endogenous control. The graph shows the mean values ± *SE* of three biological replicates. WT, wild type; EV, empty vector; L2 and L7, lines silenced for *SlMIPS1* using RNAi. Significant difference, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, NS, no significant difference.

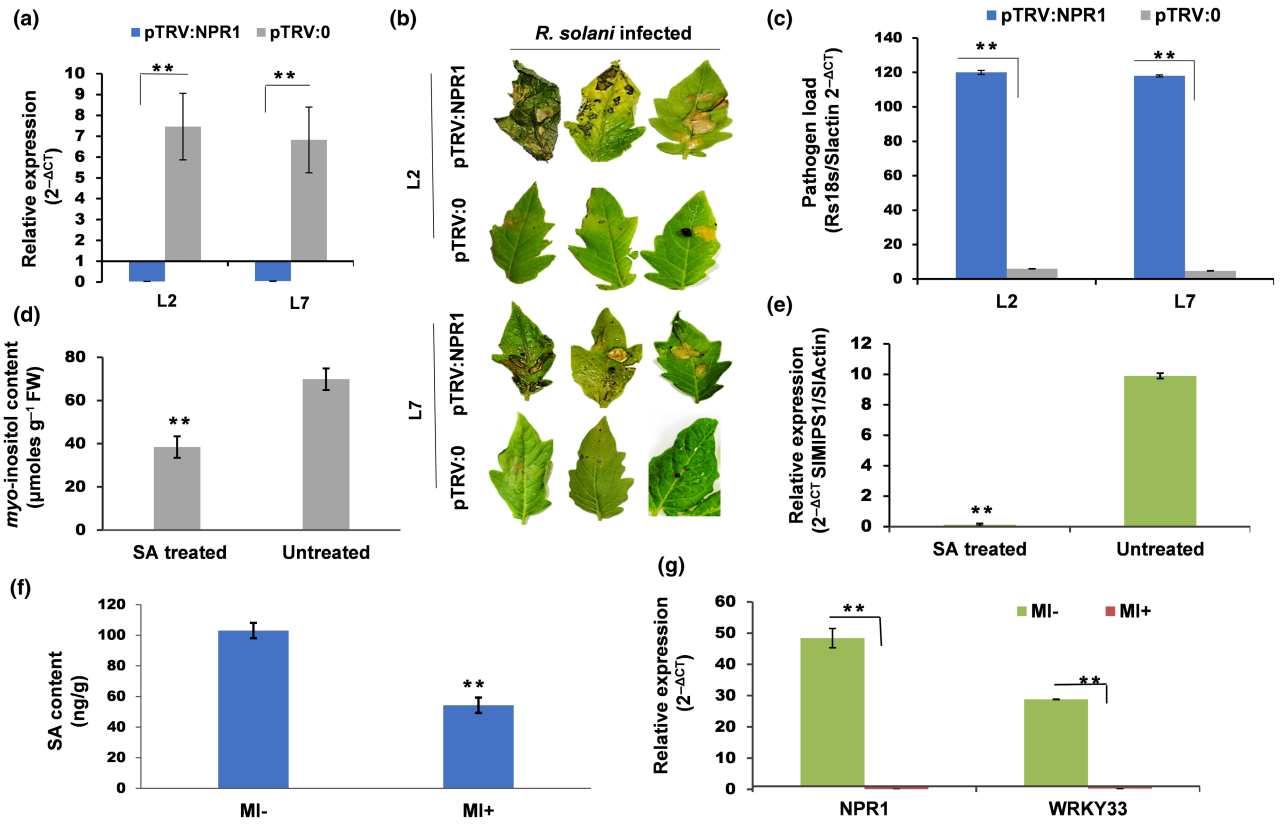

FIGURE 5 Salicyclic acid (SA) and *SlMIPS1* negatively regulate each other to mediate disease tolerance against *Rhizoctonia solani* in tomato. (a) Reverse transcription-quantitative PCR (RT-qPCR)-based expression analysis of *NPR1*, (b) disease symptoms, and (c) pathogen load in pTRV:NPR1 (*NPR1*-silenced) and pTRV:0 (control)-infiltrated *SlMIPS1*-silenced tomato plants upon *R. solani* infection at 3 days post-inoculation (dpi). (d) Gas chromatography-mass spectrometry-based quantification of *myo*-inositol content, and (e) RT-qPCR-based quantification of *SlMIPS1* expression in SA-treated and untreated tomato plants upon *R. solani* infection, at 3 dpi. (f) Liquid chromatography-mass spectrometry-based SA estimation, and (g) RT-qPCR-based quantification of *NPR1* and *WRKY33*, in tomato plants, with and without *myo*-inositol (MI) treatment, at 3 dpi. The gene expression was normalized using tomato *actin* as an endogenous control. The graph shows the mean values ± *SE* of three biological replicates. Significant difference at $^{**}p \leq 0.01$.

2.5 | SA negatively regulates *SlMIPS1* expression in tomato

We observed that exogenous SA treatment reduced *myo*-inositol content (Figure 5d), *SlMIPS1* expression (Figure 5e), and prevented *R. solani* infection (Figure S16a–c) in WT tomato. Also, exogenous SA treatment led to a significant reduction in the expression of *GUS* (*β-glucuronidase*) reporter gene under the *SlMIPS1* promoter in *Nicotiana benthamiana* (Figure S17a,b). On the other hand, *myo*-inositol (100 mM) treatment caused a reduction in SA content (Figure 5f) and downregulated expression of SA-responsive *NPR1* and *WRKY33* genes (Figure 5g) in tomato. Considering the above, we speculate that SA and *myo*-inositol negatively regulate each other to modulate host defence during *R. solani* infection. Notably, SA-responsive transcription factor binding motifs, including those of WRKY (Chen et al., 2019; Zheng et al., 2006) and TCP (Li et al., 2018; Wang et al., 2015), were present in the promoter region of *SlMIPS1* (Figure S18a) as well as *OsMIPS1* (Figure S18b). There are many TCP proteins in plants, and they interact with other TCPs as well as various SA-responsive transcription factors to modulate SA signalling (Nicolas & Cubas, 2016; Zheng et al., 2015). We selected one of the TCPs (SlTCP21) having the maximum number of potential binding motifs in the SlMIPS1 promoter, as per the PlantPAN database. The yeast one-hybrid (Y1H) assay as well as the reporter:β-glucuronidase (GUS) transactivation assay in *N. benthamiana* leaves reflected that co-expression of SlTCP21 transactivated the *SlMIPS1* promoter (Figure 6). The role of SA-responsive transcription factor(s), particularly TCPs, in modulating the interplay between SA, MIPS, and sugar signalling during the elaboration of plant defence response needs further investigation.

3 | DISCUSSION

R. solani AG1-IA is a necrotrophic polyphagous fungal pathogen, and recent studies have suggested that it adopts a brief biotrophic phase during the initial stage of plant colonization to modulate host metabolic machinery and avoid induction of a defence response (Ghosh et al., 2017; Kouzai et al., 2016; Molla et al., 2020; Pradhan et al., 2021). During the necrotrophic phase the pathogen triggers

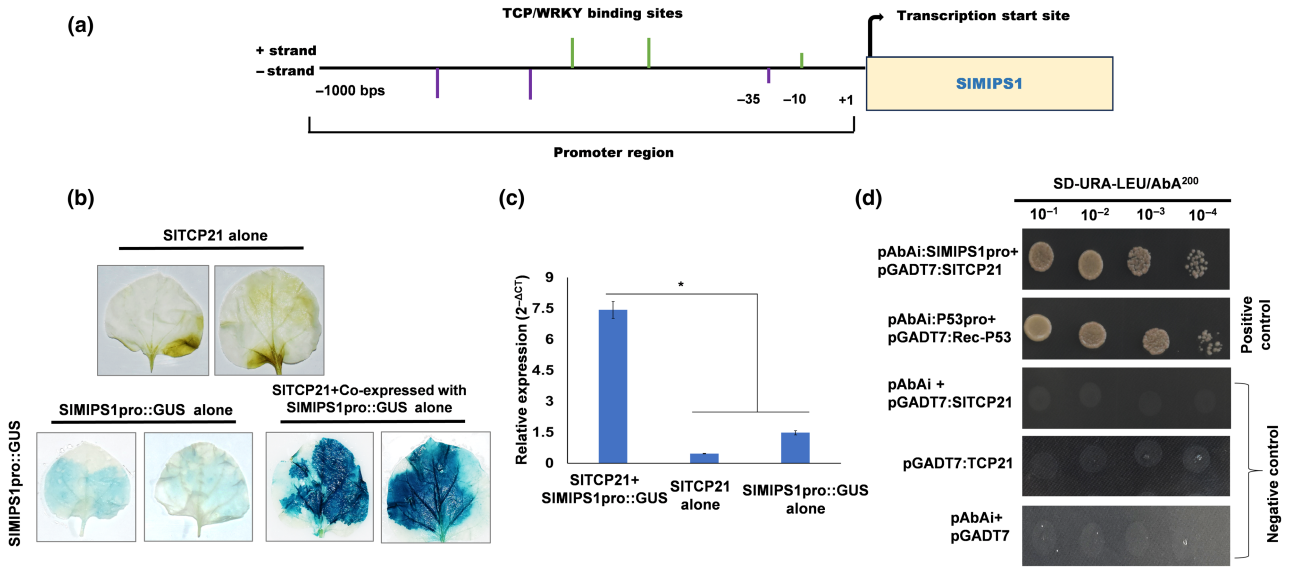

FIGURE 6 SlTCP21 interacts with the *SlMIPS1* promoter and transactivates it in yeast and *Nicotiana benthamiana*. (a) Schematic representation of potential salicyclic acid (SA)-responsive transcription factor (TCP and WRKY) binding sites in the SlMIPS1 promoter. (b) β-glucuronidase (GUS) reporter assay reflecting transactivation of the *SlMIPS1* promoter (appearance of a blue colour) upon co-expression of *SlTCP21* in *N. benthamiana* leaves. (c) The reverse transcription-quantitative PCR-based quantification of relative expression of *GUS* with respect to the reference gene (*NptII*) in *N. benthamiana*. (d) Yeast one-hybrid assay, the growth of yeast cells co-transformed with the bait (pAbAi:SlMIPS1pro) and prey (pGADT7:SlTCP21) constructs on double drop out (SD/−Leu/−Ura/+Aba) plates reflected transactivation. Similar results were obtained in three independent experiments. The graph shows the mean ± *SE* of three independent biological replicates. Significant difference using one-way analysis of variance and the Student-Newman-Keuls test, $^{*}p < 0.05$.

host cell death to feed on dead tissues. Our study emphasizes that *R. solani* enhances the expression of its own as well as the host plant *myo*-inositol-1-phosphate synthase (*MIPS1*) gene, which catalyses the rate-limiting step in *myo*-inositol biosynthesis, to promote necrotrophy. Silencing of *Rs_MIPS* compromised the pathogen growth under an oxidative-stress-enriched environment, which was restored upon exogenous *myo*-inositol treatment. As enhanced ROS accumulation is associated with the necrotrophic phase of *R. solani* infection (Das et al., 2024; Ghosh et al., 2017; Kumar et al., 2023; Oreiro et al., 2020; Wu et al., 2015), we speculate that upregulation of *Rs_MIPS* may facilitate the pathogen to better manage oxidative stress and sustain growth during plant colonization. This is supported by our observation that *Rs_MIPS*-silenced *R. solani* was compromised in causing disease, while exogenous *myo*-inositol treatment enhanced disease susceptibility.

We observed that *OsMIPS1*-silenced rice lines exhibited pleiotropic growth defects. This emphasizes the practical limitation of using *OsMIPS1* silencing as an approach to engineer sheath blight disease resistance in rice. However, SlMIPS1-silenced tomato lines showed a normal growth phenotype; therefore, we focused on characterizing the role of *SlMIPS1* during *R. solani* infection. *SlMIPS1*-silenced lines were tolerant to infection, and they exhibited enhanced SA but reduced JA levels and upregulated expression of SA-responsive genes, including *NPR1* (Nonexpressor of Pathogenesis-Related genes 1, which potentially functions as an SA receptor and regulates the homeostasis of SA/JA signalling; Dong, 2004; Kinkema et al., 2000; Sun et al., 2018), under infected conditions. As VIGS-mediated downregulation of *NPR1* rendered *SlMIPS1*-silenced lines susceptible to *R. solani* infection, we emphasize that SA-mediated defence is important for disease tolerance in *SlMIPS1*-silenced lines. It is to be noted that previous studies have demonstrated that the downregulation of *myo*-inositol induces SA-mediated defence in *Arabidopsis* (Chaouch & Noctor, 2010; Meng et al., 2009). SA plays an important role in eliciting disease tolerance against *R. solani* (Kouzai et al., 2018), and we did observe that exogenous SA treatment prevented the pathogen from establishing disease in tomato. Our study also points out that SA negatively regulates SlMIPS expression, potentially through the recruitment of SA-responsive transcription factors, particularly WRKYs and TCPs (Chen et al., 2019; Li et al., 2018; Wang et al., 2015; Zheng et al., 2006), as their binding sites are present in its promoter region. In support of this, we observed that one of the TCPs, namely SlTCP21, did transactivate the SlMIPS1 promoter. However, the role of TCP in modulating the cross-talk between SA and *SlMIPS1* during pathogen infection needs further investigation.

Being central to *myo*-inositol biosynthesis, modulation of *MIPS* expression can have a profound impact on sugar metabolism in plants (Valluru & Van den Ende, 2011). We observed that under infected conditions, expression of *LDH* (lactate dehydrogenase, which converts pyruvate into lactate) and *HK1* (hexokinase1, which converts glucose into glucose-6- phosphate) was significantly enhanced in *SlMIPS1*-silenced lines. As LDH is involved in ROS homeostasis in plants (Dolferus et al., 2008; Jain et al., 2020), we anticipated that its upregulation could facilitate better management of oxidative stress during *R. solani* infection. Moreover, considering that HK1 is associated with cell death response in plants (including *A. thaliana* and *N. benthamiana*; Bruggeman et al., 2015; Kim et al., 2006) and

induces PR genes (including *PR1*) expression in *Arabidopsis* (Xiao et al., 2000), we speculate that enhanced expression of *HK1* may promote elicitation of defence response in *SlMIPS1*-silenced lines. In accordance with the previous study that HK1 regulates trehalose biosynthesis in *Arabidopsis* (Avonce et al., 2004; Lunn et al., 2014; Ponnu et al., 2020), we observed that *SlMIPS1*-silenced lines had significantly enhanced trehalose content under infected conditions. As trehalose treatment impairs sclerotial development in *R. solani* (Wang et al., 2018) and enhances SA-mediated defence in tomato (MacIntyre et al., 2022), we anticipate that enhanced trehalose content will prevent the pathogen from colonizing *SlMIPS1*-silenced lines.

Phytopathogens, including necrotrophs, convert the infection site into sink tissue to ensure the availability of sucrose (Kanwar & Jha, 2019; Lacrampe et al., 2021). Sucrose needs to be converted into other hexoses, such as fructose, for utilization by the pathogens. HK1, along with different sugar sensors, including SUS (sucrose synthase) and SWEETs (*SlSWEET11A*, *SlSWEET11B*, *SlSWEET11C*, *SlSWEET11D*, *SlSWEET12A*, and *SlSWEET12B*), are modulated during pathogenesis to optimize the source-to-sink ratio in plants (Fatima et al., 2022; Feng et al., 2015; Kanwar & Jha, 2019; Lacrampe et al., 2021). SUS catalyses the breakdown of sucrose into fructose and uridine diphosphate glucose (UDP-glucose), leading to reduced sucrose accumulation (Cabello et al., 2014; Stein & Granot, 2019), whereas SWEET proteins facilitate the transport of sucrose from source to sink tissues (Chen et al., 2010; Kanwar & Jha, 2019; Morkunas & Ratajczak, 2014). In *SlMIPS1*-silenced lines, although the sucrose:fructose ratio was substantially reduced and *SUS* expression was enhanced, *SWEET* genes were significantly downregulated under infected conditions. The enhanced SUS will facilitate the conversion of sucrose present at the infection site into fructose as well as UDP-glucose and therefore lower the sucrose:fructose ratio. However, due to the downregulation of *SWEETs*, sucrose transport to the infection site will be hampered. This would limit sugar availability in the infected tissues and restrict the pathogen from effectively colonizing the *SlMIPS1*-silenced tomato. Previous studies have shown that modulation of *SWEET11* and *SWEET14* expression affects *R. solani* infection in rice (Chen et al., 2010; Gao et al., 2018; Kim et al., 2021; Singh et al., 2024). In *Arabidopsis*, a mutation in *AtSWEET11/12* enhances SA accumulation and provides tolerance against *Colletotrichum higginsianum*, a hemibiotrophic fungal pathogen (Gebauer et al., 2017). This suggests that downregulated expression of *SWEETs* may also contribute to defence against *R. solani* in *SlMIPS1*-silenced lines.

Overall, our study points out that *SlMIPS1* and SA negatively regulate each other to modulate the *R. solani* infection. We emphasize that upregulation of *SlMIPS1* is beneficial for *R. solani* to ensure the availability of *myo*-inositol, modulate the conversion of source to sink tissues, and prevent SA-mediated defence during plant colonization. In *SlMIPS1*-silenced tomato lines, due to the downregulated expression of *SWEETs*, sugar availability is restricted at the infection site, and SA-mediated defence is triggered, which prevents infection. Further studies are required to identify additional factors, particularly the transcriptional regulators that are involved in the interplay of *SlMIPS1*, *myo*-inositol (as well as other sugars), *SWEETs*, and SA during *R. solani* infection.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant material and growth conditions

Rice (*O. sativa* subsp. *indica* 'PB1' and 'IR64') and tomato (*S. lycopersicum* 'Pusa Ruby') plants were grown in pots under greenhouse conditions (80% humidity and 16/8 h day/night) at 28°C and 25°C, respectively. *A. thaliana* (Col 0) plants were grown in a growth chamber (22°C, 60% humidity) on Soilrite, initially for 4 weeks under short-day conditions (8/16 h day/night) and subsequently under long-day conditions (16/8 h day/night) until flowering and seed maturation.

4.2 | *R. solani* growth and infection assay

R. solani AG1-IA strain BRS1 (Francis et al., 2023; Ghosh et al., 2021) was cultured on potato dextrose agar (PDA; Himedia) plates at 28°C. The growth of *R. solani* sclerotia (collected from PDA plates) was analysed on minimal medium (Czapek Dox agar, CDA) plates with and without supplementation of various chemicals (1 mM *myo*-inositol, 1 mM D-sorbitol, and 100 μM hydrogen peroxide). The experiment was independently repeated three times, using at least four sclerotia per treatment in each replicate.

For the pathogenicity assay, equal-sized sclerotia were used for infecting rice (cv. PB1), tomato (cv. Pusa Ruby), and *A. thaliana* (Col-0), as described in Kant et al. (2019), Swain et al. (2019), and Chandan, Kumar, Swain, et al. (2023). Briefly, sclerotia were inoculated into the inner side of rice sheaths while they were attached to the leaves of tomato/*A. thaliana*. The plants were incubated in an infection chamber under controlled conditions and they were regularly sprayed with water to maintain high humidity. The disease symptoms (necrotic lesions) were recorded at different time points. As reported previously, the relative vertical sheath colonization (RVSC) index was estimated for quantitative analysis of disease index in rice, while in tomato/*A. thaliana*, the lesion area in the leaves was quantified using ImageJ (http://rsbweb.nih.gov/ij). The experiment was independently repeated three times, using at least four plants per replicate. A minimum of four tillers per rice plant and four leaves per tomato/*Arabidopsis* plant were analysed in each experiment.

4.3 | Identification and phylogenetic analysis of MIPS homologues in plant and fungal species

OsMIPS1 (LOC_Os03g09250) gene sequence was used as a query for BLAST search in the Rice Genome Annotation Project database

(RGAP; http://rice.uga.edu) (Sakai et al., 2013) and the Sol Genomic network database (https://solgenomics.net) (Fernandez-Pozo et al., 2015) to identify different homologues in rice and tomato, respectively. BLASTp searches in the NCBI database were conducted using OsMIPS1 (*Oryza sativa* MIPSs), AtMIPS1 (*Arabidopsis thaliana* MIPS1), and SlMIPS1 (*Solanum lycopersicum* MIPS1) as queries to identify MIPS homologues in other plant species. *R. solani MIPS* (*Rs_MIPS*; Rs_10081.1; XM_043329844.1) sequence was retrieved from the rice sheath blight (RSB) database (https://nipgr.ac.in/RSB/) (Francis et al., 2023) and was used to retrieve MIPS homologues from other fungi using the NCBI database. The amino acid sequences of plant and fungal MIPS were aligned using ClustalW. A phylogenetic tree was constructed using MEGA X software (Kumar et al., 2018) by maximum likelihood with 500 bootstrap values (cut-off 50%) and the Tamura–Nei model, as described earlier (Ghosh et al., 2019).

4.4 | dsRNA-mediated silencing of *R. solani* genes

A unique gene fragment (181 bp) of *Rs_MIPS* having no off-target silencing effect was identified using siRNA-Finder (si-Fi) (http://labtools.ipk-gatersleben.de/) (Lück et al., 2019). The fragment was PCR amplified from *R. solani* cDNA using a gene-specific primer pair with a flanking T7 promoter sequence on the 5′ end of both primers (Table S1). In-vitro transcription reactions were set up to synthesize gene-specific dsRNA using the MEGAscript T7 Transcription Kit (Thermo-Scientific), as described in Ghosh et al. (2021). *Rs_GT34* (*R. solani glycosyl transferase family 34*) was used as a negative control (Francis et al., 2023; Ghosh et al., 2021). *R. solani* sclerotia were treated with buffer or dsRNA (targeting *Rs_MIPS/Rs_GT34*) for 24 h at 28°C and used to infect rice/tomato plants. The plants were monitored for the appearance of disease symptoms. At least five plants (four leaves/sheaths per plant) were infected per treatment, and each experiment was independently repeated three times.

4.5 | RNAi construct for silencing of rice/tomato *MIPS1* genes

The 3′ untranslated region (320 bp) of the *OsMIPS1* gene with no predicted off-targets was selected by siRNA-Finder (si-Fi) (http://labtools.ipk-gatersleben.de/) software (Lück et al., 2019). As the selected region showed 85% similarity with that of *SlMIPS1*, we used the same RNAi construct for silencing *OsMIPS1* in rice and *SlMIPS1* in tomato. The sense as well as antisense fragments of the target region were PCR amplified from rice DNA using gene-specific primers (Table S1) and cloned into a modified pGEM-T Easy vector (having an intron sequence of *Brassica*). Further, the sense-intron-antisense cassette was subcloned into the pBI121 binary vector to generate a hairpin RNAi construct (pBI121:MIPS1). The construct was mobilized into *Agrobacterium tumefaciens* LBA4404 and GV3101 for transformation in rice and tomato, respectively.

4.6 | Generation of stable RNAi lines in rice and tomato

Callus (30 days old) of rice cv. IR64 (Swain et al., 2019) and cotyledon of 10-day-old tomato (cv. Pusa Ruby) seedlings (Chandan, Kumar, Kabyashree, et al., 2023) were used as explants. Upon co-cultivation with *A. tumefaciens* (LBA4404 for rice or GV3101 for tomato) harbouring pBI121:*MIPS1*, the explants were incubated under dark conditions for 48 h and subcultured at a 15-day interval in kanamycin-containing selection medium. Subsequently, explants were shifted to shoot regeneration medium and thereafter transferred into rooting medium, as described before (Chandan, Kumar, Kabyashree, et al., 2023; Swain et al., 2019). The positive transgenic plants were selected at T_0 generation by PCR using intron-specific and neomycin phosphotransferase (NPT-II) gene primers (Table S1). *OsMIPS1*-silenced RNAi rice lines exhibited severe growth defects and could not be propagated beyond T_2 generation. On the other hand, *SlMIPS1*-silenced RNAi tomato lines exhibited normal growth and yielded viable seeds.

4.7 | Heterologous overexpression of *SlMIPS1* in *atmips1* mutant *A. thaliana*

The SlMIPS1 gene (1533 bp; Solyc04g054740.2) was PCR amplified from tomato cDNA and cloned in the pGEM-T Easy vector. It was subcloned into the pBI121 vector using restriction digestion (XbaI and SacI)-based cloning. The construct was mobilized into *A. tumefaciens* GV3101 and used for transformation into *atmips1* mutant *A. thaliana* (*atmips1*; Salk id: SALK_021779; Donahue et al., 2010) by the floral dipping method (Chandan, Kumar, Swain, et al., 2023; Clough & Bent, 1998). The transformants were selected on kanamycin-containing Murashige and Skoog (MS; Himedia) medium. Positive transformants were confirmed by PCR using *SlMIPS1*-specific primer pairs (Table S1) and propagated to the T_3 generation for further analysis.

4.8 | RT-qPCR-based expression analysis

Total RNA was extracted from the leaf samples using the RNeasy plant mini kit (Qiagen), and cDNA was synthesized using the verso cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed using gene-specific primers (Table S1). Fold change was estimated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), and relative expression was calculated by the $2^{-\Delta Ct}$ method, wherein ΔCt is the difference between the C_t value of the target and reference gene, as described before (Chandan, Kumar, Swain, et al., 2023). Each experiment was repeated using three technical and three biological replicates.

4.9 | Pathogen load quantification

R. solani biomass was estimated as the abundance of 18S rRNA in the infected plants, using the $2^{-\Delta Ct}$ method, wherein ΔCt is the difference

between C_t values of fungal 18S rRNA (target) and endogenous genes (18S rRNA for rice, *actin* for tomato), as described in Ghosh et al. (2021). Each experiment was repeated three times, using three technical and biological replicates.

4.10 | Histochemical staining

3,3′-diaminobenzidine (DAB) histochemical staining was used to detect H_2O_2 accumulation in rice and tomato, as described in Ghosh et al. (2017) and Chandan et al. (2019). Briefly, samples were stained with 1 mg/mL of DAB solution (prepared in 50 mM Tris-acetate buffer, pH 5.0) and incubated in the dark at 25°C for 16 h with intermittent shaking. Upon destaining with an ethanol and acetic acid (3:1 ratio) solution for 3–4 h, the appearance of brown patches due to H_2O_2-mediated oxidation of DAB was photographed, using a Nikon DS-Rs1 camera. The experiment was repeated three times, with a minimum of 10 tomato leaves or rice sheaths being analysed per replicate.

4.11 | GC-MS-based estimation of sugars

GC-MS was used for the quantification of different sugars (*myo*-inositol, trehalose, sucrose, and fructose). Briefly, 300 mg of leaf tissues were crushed in liquid nitrogen, and 1.5 mL of methanol (HPLC grade) was added immediately. The extraction was performed at 70°C for 15 min. After the addition of 1.4 mL of water and 750 μL of chloroform, samples were centrifuged at 2200*g* for 5 min. The supernatant was collected and vacuum-dried for 8–12 h using a speed vac at 40°C. For derivatization (methoxymation and trimethylsilylation), 40 μL of methoxyamine hydrochloride (20 mg/mL in pyridine) was added to each sample, and upon incubation at 37°C for 90 min, 60 μL of MSTFA (Sigma Aldrich) was added and further incubated at 37°C for 30 min (Ghosh et al., 2017; Salvi et al., 2016). Two microlitres of sample was used for GC-MS analysis employing a Shimadzu GC-MS-QP2010 coupled with an autosampler-auto injector (AOC-20si) using an Rtx-5 capillary column (Restek Corp.) and helium as carrier gas. The following programme, 80°C isothermal heating for 2 min, followed by a ramp rate of 5°C/min to 250°C, a withhold of 2 min, and a final ramp of 10°C/min, a withhold time of 24 min, was used to run each sample. The chromatogram integration and mass spectra analysis of derivatized metabolites were performed through GC-MS solution software (Shimadzu) and further validated using authentic standards, as described in Kundu et al. (2018). The experiment was repeated three times, with a minimum of five biological and technical replicates per sample.

4.12 | LC-MS/MS-based quantification of defence-related phytohormones

The leaf tissues (200–300 mg) were harvested during the day and ground into a fine powder using a mortar pestle. The powder was suspended in 1 mL of methanol containing 60 ng of 9,10-d2-9,10-dihydrojasmonic acid, 60 ng of D4-salicylic acid, 60 ng of D6-ABA (Santa Cruz Biotechnology), and 15 ng of JA-[13C6] Ile conjugate as an internal standard. Homogenate was vortexed for 2 min and incubated for 20 min, at 4°C, with gentle shaking. After centrifugation at 20,817 × *g* for 15 min at 4°C, the supernatant was collected and the pellet was further extracted using 500 μL of methanol. The extracted supernatants were pooled, evaporated in a speed vac at 30°C, and dissolved in 500 μL of methanol. Chromatography was performed on a 1200 HPLC system (Agilent Technologies) using a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm; Agilent). The detailed protocol for phytohormone estimation was described earlier (Vadassery et al., 2012). The experiment was repeated three times, with a minimum of five biological and technical replicates per sample.

4.13 | Exogenous treatment with SA and *myo*-inositol

Different concentrations (1 mM, 2 mM, and 5 mM) of SA were prepared in deionized water with 0.1% Tween 20 (surfactant) and sprayed onto 40-day-old tomato plants. SA at 1 mM was optimal in imparting disease tolerance, as at higher concentrations yellowing of leaves was observed. Similarly, different concentrations (25 mM, 50 mM, 100 mM, and 200 mM) of *myo*-inositol were sprayed onto plants, and 100 mM solution were standardized for subsequent study. D-sorbitol (100 mM) was used as an osmolyte control. The experiment was independently repeated three times using four or five leaves per plant and a minimum of four plants per treatment per replicate.

4.14 | VIGS in tomato

Phytoene desaturase (*PDS*, a carotenoid biosynthetic gene that upon silencing, gives a photobleaching phenotype in tomato) was used as a positive control for VIGS in tomato, as described previously (Chandan et al., 2019; Chandan, Kumar, Kabyashree, et al., 2023; Kant et al., 2019; Marino et al., 2012). The partial gene fragments of *PDS* (374 bp; accession: NM_001247166.2) and *NPR1* (350 bp; accession: NM_001247629.2) were PCR amplified from tomato cDNA using gene-specific primers (Table S1) and cloned into the pTRV2 vector at XbaI/KpnI restriction sites. The constructs were transformed into *A. tumefaciens* GV3101, grown in YEP broth, and pelleted at 2655 × *g* for 5 min. The agrobacterial cells harbouring pTRV2 plasmids were resuspended in induction buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone) at $OD_{600} = 1$ and incubated at 28°C for 4 h. Subsequently, the suspension was mixed with pTRV1 harbouring agrobacterial cell suspension in 1:1 and infiltrated into 10-day-old tomato seedlings (using a 1 mL needleless syringe) as described before (Chandan et al., 2019; Kant et al., 2019). The plants were incubated under greenhouse conditions for 3–4 weeks,

thereafter subjected to *R. solani* infection, and monitored for the appearance of disease symptoms. Each experiment was repeated three times, with a minimum of 10 tomato seedlings per gene construct per replicate.

4.15 | Promoter:GUS reporter assay

PlantPAN 3.0 (http://plantpan.itps.ncku.edu.tw.) was used to identify SA-responsive transcription factor (TCP and WRKY) binding sites in the promoter region of *OsMIPS1* and *SlMIPS1*. The *SlMIPS1* promoter, enriched in TCP/WRKY binding motifs, was identified using the PLAZA 4.0 database (https://bioinformatics.psb.ugent.be/plaza/). It was PCR amplified from tomato and cloned in a promoterless binary vector, pBI101, upstream of *GUS*, using SmaI and SacI restriction enzymes. The *SlMIPS1p*::GUS construct was immobilized in *A. tumefaciens* GV3101 and infiltrated into the abaxial surface of 15-day-old tomato leaves. Considering the difficulty of agroinfiltration in tomato, attempts were made to infiltrate at least two places in each leaf. GUS expression was analysed with and without *R. solani* infection (3 dpi) using a histochemical GUS assay (Chandan, Kumar, Swain, et al., 2023). Briefly, leaves were incubated in an X-gluc solution containing 20 mg of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5% Triton X-100, 0.1 M potassium ferrocyanide and potassium ferricyanide, and 50 mM sodium phosphate buffer (pH 7). Upon overnight incubation at 37°C, samples were destained in an ethanol and acetic acid (3:1) solution. Samples were visualized under a white-light transilluminator, and images were captured using a digital camera (Nikon DS-Rs1). Expression of the *GUS* gene was quantified by RT-qPCR using a gene-specific primer pair (Table S1). The experiment was repeated three times, with a minimum of three biological repeats.

To analyse the effect of exogenous SA treatment on the expression of the *SlMIPS1* promoter, *SlMIPS1*pro::GUS construct was infiltrated into the abaxial surface of 10-day-old *N. benthamiana* leaves. The leaves were further subjected to SA treatment (1 mM), and promoter activity was analysed through quantification of GUS expression, as mentioned above.

4.16 | Y1H assay

The *SlMIPS1* promoter was cloned in the pAbAi bait vector (Clontech), upstream to the aureobasidin A (*AbA*) gene, and after linearization with the BstBI enzyme, it was transformed into the Y1H Gold yeast strain (using EZ-Yeast Transformation Kit; MP Biomedicals). The positive transformants (pAbAi:SlMIPS1; Y1H-Bait) were selected on AbA (200 mg/mL) containing SD − Ura plates. Subsequently, the *SlTCP21* (Solyc03g006800.1) gene was PCR amplified from tomato cDNA and cloned in a pGADT7 (Clontech) prey vector (pGADT7:SlTCP21; Y1H-Prey) as a GAL4 transcription activation domain fusion protein. It was transformed into the Y1H Bait strain (pAbAi:SlMIPS1), and the positive interaction between prey and bait constructs was tested on double drop-out (SD − Ura − Leu) plates containing AbA (200 mg/mL), as described in Chandan, Kumar, Swain, et al. (2023).

4.17 | Transactivation of *SlMIPS1* promoter by SlTCP21

SlTCP21 was PCR amplified from tomato cDNA, cloned in a pGJ100 binary vector (Chandan, Kumar, Swain, et al., 2023), and transformed into *A. tumefaciens* GV3101. Similarly, the *SlMIPS1*pro::GUS construct was mobilized into *A. tumefaciens* GV3101. The agrobacterial strains were co-infiltrated into *N. benthamiana* leaves and the appearance of GUS was monitored after 48 h by staining the leaves in X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) solution (1 mg/mL) for 16 h at 37°C (Chandan, Kumar, Swain, et al., 2023). After 3–4 h of destaining (1:3 ratio of glacial acetic acid:ethanol), the leaves were photographed. RT-qPCR was used to quantify the *GUS* expression in *N. benthamiana* leaves infiltrated with the *SlMIPS1*pro::GUS and *SlTCP21* construct.

4.18 | Statistical analysis

Each experiment was independently repeated three times using three technical replicates. Values are expressed as the mean ± *SE*. SPSS-17 statistical software (SPSS Inc.) was used to perform one-way analysis of variance followed by Newman-Keuls tests. The significant difference with $p < 0.05$ is indicated as * while $p < 0.01$ is indicated as **.

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CONFLICT OF INTEREST STATEMENT

No conflict of interest declared.

DATA AVAILABILITY STATEMENT

All data supporting the study are available within the article and within its supplemental material.

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SUPPORTING INFORMATION

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