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Lasiodiplodia theobromae protein LtScp1 contributes to fungal virulence and protects fungal mycelia against hydrolysis by grapevine chitinase

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Abstract

The LysM proteins have been reported to be important for the virulence and host immunity suppression in herbaceous plant pathogens, whereas far less information is documented in the woody plant pathogen *Lasiodiplodia theobromae*. To investigate the functional mechanism of LysM protein in *L. theobromae*, one gene *LtScp1* was cloned and characterized detailedly in the current study. Transcription profiling revealed that *LtScp1* was highly expressed at the infectious stages. Compared to wild type, overexpression and silencing of *LtScp1* in *L. theobromae* led to significantly increased and decreased lesion areas, respectively. Moreover, LtScp1 was determined to be a secreted protein via a yeast signal peptide trapping system. Interestingly, LtScp1 was confirmed to be modified by the *N*-glycosylation, which is necessary for the homodimerization of LtScp1 molecules. Furthermore, it was found that LtScp1 interacted with the grapevine chitinase VvChi4 and interfered the ability of VvChi4 to bind chitin. Collectively, these results suggest that LtScp1 functions as a virulence factor to protect the fungus from degradation during the infection.

INTRODUCTION

Plants are subjected to numerous abiotic and biotic stressors throughout their life histories. It is accepted that plants have developed two crosstalk lines of defences to prevent pathogen invasion and colonization inside host tissues (de Wit, 2007; Jones & Dangl, 2006; Yuan et al., 2021). The first line provides basal defence and is based on the recognition of conserved microbial features, known as pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin, elongation factor thermo unstable (EF-Tu), lipopolysaccharide (LPS), peptidoglycan (PGN), fungal chitin, and oomycete glucans, by pattern recognition receptors (PRRs) (Boller & He, 2009; Newman et al., 2013; Thomma et al., 2011; Win et al., 2012). The recognition process is associated with a series of immune responses, including callose deposition, lignin formation, stomata closure, antimicrobial compound production, reactive oxygen species (ROS) generation, defence-related gene activation and signal transduction events, which are generally termed PAMP-triggered immunity (PTI) (Couto & Zipfel, 2016; Han & Kahmann, 2019; Macho & Zipfel, 2014; Newman et al., 2013). This defence response is relatively weak but broad-spectrum (Wang et al., 2014). Although PTI responses are prevalent in plants, successful pathogens can surmount PTI by secreting effectors to

suppress the PTI response. As coevolution proceeded, plants also evolved sophisticated recognition systems to detect these effectors in a highly specific manner to activate the second line of immunity, which is accompanied by ROS accumulation, hypersensitive cell death, systemic acquired resistance and defence-related gene activation and is referred to as effector-triggered immunity (ETI) (Jones & Dangl, 2006; Thomma et al., 2011). Generally, the ETI response is more robust, effective, prolonged, and rapid than basal defence (Newman et al., 2013; Thomma et al., 2011; Wang et al., 2014). These responses trigger a series of coevolutionary arm races between pathogens and plants, during which the pathogens respond by deleting or mutating the recognized effector gene or by generating novel effectors to avoid or suppress ETI. Plants, in turn, develop new resistance proteins to mediate effector recognition processes (de Wit, 2007; Jones & Dangl, 2006; Stergiopoulos & de Wit, 2009). Up to now, hundreds of effector proteins have been characterized in various plant pathogens, including fungi, oomycetes, bacteria, and nematodes, and these studies reveal that effector proteins play multiple and significant roles during plant–microbe interaction (Davis et al., 2008; Galán & Collmer, 1999; Galán & Wolf-Watz, 2006; Hogenhout et al., 2009; Lo Presti et al., 2015; Selin et al., 2016; Stergiopoulos & de Wit, 2009).

The lysin motif (LysM) was first discovered in the lysozyme of bacteriophage *Bacillus* phage *Φ*29, where it was detected as a C-terminal repeat composed of 44 amino acids (Buist et al., 2008; Garvey et al., 1986). Afterwards, LysM was identified in many bacterial and eukaryotic proteins, and nowadays they have been commonly found in all kingdoms of life (Akcapinar et al., 2015; Buist et al., 2008; Liu et al., 2019). The LysM was originally described as a small carbohydrate-binding domain that binds to *N*-acetylglucosamine (GlcNAc)-containing carbohydrates, such as chitin, chitiooligosaccharides and peptidoglycan (Akcapinar et al., 2015; Buist et al., 2008; Liu et al., 2019). In the Carbohydrate-Active enZymes (CAZy) database (Lombard et al., 2014), LysMs were widely identified in different protein types including glycoside hydrolases, transglycosylases, peptidases, amidases, chitinases, receptor-like kinases, and effectors (Akcapinar et al., 2015). In plant pathogens, previously characterized LysM-containing proteins, such as Ecp6 of *Cladosporium fulvum* (de Jonge et al., 2010; Sánchez-Vallet et al., 2013), Slp1 of *Magnaporthe oryzae* (Mentlak et al., 2012), ChELP1 and ChELP2 of *Colletotrichum higginsianum* (Takahara et al., 2016), Vd2LysM of *Verticillium dahlia* (Kombrink et al., 2017), Mg3LysM, Mg1LysM, and MgxLysM of *Zymoseptoria tritici* (Marshall et al., 2011; Tian et al., 2021), functioned as effectors to protect fungal hyphae from being degraded or suppress chitin-triggered plant immunity. The biological function of LysM protein in opportunistic

plant pathogen *Lasiodiplodia theobromae*, however, is barely documented.

Lasiodiplodia theobromae, a Botryosphaeriaceae family member, has been recognized as a notorious woody plant pathogen that is capable of infecting over 500 host species, including fruit and nut trees, vegetable crops, and ornamental plants. This pathogen causes canker, dieback, and fruit and root rot diseases, thereby resulting in considerable crop losses on a global scale (Úrbez-Torres et al., 2008; Yan et al., 2013a, 2013b). *L. theobromae* was first reported to be able to cause vascular cankers and grapevine dieback in Egypt (El-Goorani et al., 1972). The fungus obtains nutrition from host tissues and maintains a latent lifestyle inside host plants for a long period of time under suitable environmental conditions. External stimuli, such as host behaviour changes, microbial equilibrium disruption, and extreme environmental events, result in the fungal lifestyle changing from endophytic to pathogenic, making this organism an opportunistic plant pathogen (Chethana et al., 2016; Slippers and Wingfield, 2007). Due to its economic importance in controlling the disease caused by *L. theobromae*, this fungus has attracted increasing attention from pathologists and orchardists. Additionally, the fungus has been regarded as a model organism for investigating the interaction between opportunistic plant pathogens and woody plants. To date, research on the Botryosphaeriaceae family has mainly focused on fungal isolation and identification, disease symptom description, epidemiology investigation, disease prevention and control (Carlucci et al., 2015; Reis et al., 2019; Songy et al., 2019; Yan et al., 2013). Recently, comparative genome and transcriptome analyses of *L. theobromae* and five other Botryosphaeriaceae pathogens were performed by Yan et al. (2018). A total of over 350 effectors were identified in the *L. theobromae* secretome, and ~200 *L. theobromae*-specific effectors were predicted and grouped based on their functional annotations. The functional mechanisms of these effectors remain unrevealed, although a small number of effectors have been found to affect cell death in *Nicotiana benthamiana* (Yan et al., 2018).

To get a better understanding of the roles of *L. theobromae* effectors, a LysM-containing protein, LtScp1, was functionally characterized in this study. LtScp1 was confirmed to be a secreted protein by a yeast signal peptide trapping system and contributed to the virulence of *L. theobromae*. Compared to wild type, overexpressed transformants and silenced transformants of *LtScp1* displayed increased and attenuated virulence on grapevines, respectively. Furthermore, LtScp1 was found to contain three *N*-glycosylation sites, which affected the homodimerization of LtScp1 molecules. Interestingly, LtScp1 was confirmed to interact with a grapevine chitinase, VvChi4, and interfered

the ability of VvChi4 to bind chitin. Altogether, these results indicate that LtScp1 protein contributes to the virulence of *L. theobromae* and may be able to affect the chitinase activity of VvChi4 by direct interaction.

EXPERIMENTAL PROCEDURES

Strains, plant materials and culture conditions

The *L. theobromae* strains were cultured on complete media (6 g yeast extract, 3 g casein acid hydrolysate, 3 g casein enzymatic hydrolysate, and 10 g sucrose) at 25°C. Yeast strain YTK12 and the resultant transformants were cultured on CMD-W (0.67% yeast nitrogen without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar), and YPRAA (1% yeast extract, 2% peptone, 2% raffinose, 2 μg/ml antimycin A, and 2% agar) media. The yeast strain AH109 was cultured on YPDA (1% yeast extract, 2% peptone, 2% glucose, and 0.003% adenine hemisulfate) medium. The *Agrobacterium tumefaciens* strain GV3101 and *E. coli* strain BL21 were cultured on LB media (5 g yeast extract, 10 g tryptone, and 10 g NaCl). The *N. benthamiana* was grown inside a growth chamber at 25°C under a 14 h:10 h light: dark photoperiod. The green shoots of *Vitis vinifera* cv 'Summer Black' were collected from Xiangyi field vineyard in Shunyi, China.

RNA extraction and quantitative real-time polymerase chain reaction

The *L. theobromae* mycelial plugs (5 mm in diameter) were inoculated on the wounded shoots of 1-year-old susceptible cultivar *V. vinifera* cv. 'Summer Black', followed by incubation in a chamber with constant humidity and temperature. Diseased tissues were harvested at 0, 12, 36, 48, 60, and 72 h post inoculation (hpi) and used for subsequent RNA isolation. Total RNA was extracted using an RNA isolation kit according to the manufacturer's instructions (Aidlab Biotech, Beijing, China). Isolated RNA was reversely transcribed into cDNA with the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). qRT-PCR assays were performed using an ABI 7500 Real Time system (Applied Biosystems, Waltham, MA, USA) and conducted in a 16 μl volume mixture composed of 8 μl RealStar Green Fast Mixture with ROX II (GenStar Biosolutions, Beijing, China), 1.0 μl cDNA, 0.2 μM primer, and 6.4 μl sterile ddH$_2$O. The PCR programme was as follows: denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The *actin* gene was used as the internal control. Expression data were normalized to the *actin* gene and calibrated against the transcript level at 0 hpi. The relative abundance of transcripts was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). All experiments were repeated at least twice independently with three replicates each. All the primers used for qRT-PCR assays are listed in Table S2.

Pathogenicity test on detached grapevine shoots

For overexpression transformation, the *LtScp1* ORF was amplified with primer pair OE-f/OE-r (Table S2) and then cloned into the modified *pKSNTP* vector. Afterwards, the fusion construct, referred to as *pKSNTP:LtScp1*, was transformed into *L. theobromae* protoplasts using the polyethylene glycol (PEG)-mediated transformation method described by Yan et al. (2018). The resultant transformants were screened against neomycin resistance and verified by qRT-PCR. Next, two positive transformants, referred to as LtScp1-OE1 and LtScp1-OE2, were selected for pathogenicity tests. Mycelial plugs of the overexpressed transformants LtScp1-OE1 and LtScp1-OE2, as well as wild-type CSS-01s (5 mm in diameter), were collected and inoculated on wounded shoots of 1-year-old susceptible *Vitis vinifera* cv. 'Summer Black.' The inoculated grapevine shoots were placed in a chamber under constant humidity and temperature. At least five biological replicates were carried out for each overexpressed transformant.

For RNAi transformation, we amplified the sense fragment with the primer pair RNAi-Sf/RNAi-Sr and the antisense fragment with the primer pair RNAi-ASf/RNAi-ASr (Table S2) and then ligated both fragments into the *pRTN* vector in the given order. Subsequently, the fusion vector, named *pRTN:LtScp1*, was transformed into *L. theobromae* protoplasts with methods similar to those used for overexpression transformation. The protocols used for pathogenicity tests of silenced transformants were also similar to those of overexpressed transformants.

Functional validation of the LtScp1 signal peptide

Functional validation of the LtScp1 signal peptide was performed with an elegant yeast signal peptide trapping system (Fang et al., 2016; Gu et al., 2011; Jacobs et al., 1997; Oh et al., 2009). In the system, the signal peptide trapping vector *pSUC2T7M13ORI* (*pSUC2*) carries a truncated invertase gene *SUC2* that lacks its own initiating methionine codon and signal peptide coding sequence. The predicted coding sequence of LtScp1 signal peptide was amplified with the specific

primer pairs (Table S2) and then cloned into the *pSUC2* vector. The fusion construct, named *pSUC2: LtScp1*, was transformed into the invertase secretion-defective yeast strain YTK12. The resultant yeast transformants were streaked on CMD-W and YPRAA media to test for their invertase secretion abilities. Yeast cells expressing the N-terminal amino acid sequences of Avr1b from *P. sojae* and Mg87 from *M. oryzae* were used as positive and negative controls, respectively.

A. tumefaciens-mediated transient expression

The full-length ORF of *LtScp1* was amplified and ligated into the *35S*-driven expression plasmid *pGWB*. The resultant fusion construct, referred to as *pGWB: LtScp1*, was transformed into *A. tumefaciens* GV3101 using a freeze–thaw method (An et al., 1989). The resulting positive transformants were confirmed by PCR. After overnight culturing, *A. tumefaciens* was harvested by centrifugation at 4000 rpm for 10 min and washed three times with sterile ddH_2O. Subsequently, *A. tumefaciens* was resuspended in infiltration buffer (10 mM MES, pH 5.7, 10 mM $MgCl_2$, and 150 μM acetosyringone) with an optical density of 0.5 at 600 nm. After standing for 3 h at room temperature, the *A. tumefaciens* suspensions were infiltrated into 4-week-old *N. benthamiana* leaves with a needless syringe.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed using the PEG/LiAc method. A series of truncated fragments of *VvChi4* were amplified with the specific primer pairs (Table S2) and then subcloned into the *pGADT7* prey vector. The ORF of *LtScp1* was amplified and ligated into the *pGBKT7* bait vector. Both the prey and bait constructs were transferred into yeast strain AH109 simultaneously. The resultant yeast transformants were examined for growth and *β*-galactosidase activities on SD-Leu-Trp-His-Ade media.

Bimolecular fluorescence complementation assay

bimolecular fluorescence complementation (BiFC) assays were performed using the *p35S-nYFP* and *p35S-cYFP* vectors. The ORF of *LtScp1* was cloned into *p35S-cYFP* at *Bam*H I site and the ORF of *VvChi4* was cloned into *p35S-nYFP* at *Bam*H I site. The *A. tumefaciens* strain C58C1 carrying the indicated construct were coinfiltrated into 4-week-old *N. benthamiana* leaves. The fluorescence was detected at 48 h post infiltration (hpi) using a confocal laser scanning microscope.

MBP pull down and chitin pull down

The ORFs of *VvChi4* and *LtScp1* were cloned into the modified expression vector *pPICZ* and the two fusion constructs were transformed into *Pichia pastoris* X-33 for further protein expression. The expressed recombinant proteins were used for chitin pull down assays using the methods documented by Liu, Li, et al. (2012) and Liu, Liu, et al. (2012).

Sequence homology analyses

Homology analyses were performed using the sequences obtained from BLASTP against the NCBI database with LtScp1 as the query sequence. Multiple sequence alignments were generated using ClustalX2, and identical amino acids were shaded using Jalview (Mafurah et al., 2015; Mentlak et al., 2012; Takahara et al., 2016).

RESULTS

LtScp1 is a putative LysM protein

To investigate the function of putative LysM proteins in *L. theobromae*, we performed a BLASTP search against the *L. theobromae* protein database with the amino acid sequence of Ecp6 as a query (Bolton et al., 2008). Based on BLASTP analyses, a total of eight proteins were returned and one protein that shared the highest amino acid identity with Ecp6 was selected for subsequent study. Functional annotation revealed that this homologue in *L. theobromae* is a putative secreted carbohydrate-binding module family 50 protein (LtScp1). Moreover, LtScp1 was predicted to be a glycoprotein with five *N*-glycosylation sites located at Asn^{33} (NGT), Asn^{72} (NLT), Asn^{96} (NTS), Asn^{133} (NIT), and Asn^{170} (NIS) using the N-GlycoSite tool and contain a signal peptide of 20 amino acids using the SignalP, iPSORT, and PSORTII programmes (Figure S1A). Full-length amino acid sequence analyses using the Pfam programme showed that LtScp1 contains three LysM domains (Figure S1B). Next, amino acid sequences of all three LysM domains were aligned to investigate the sequence similarities among them (Figure S1C). Because Ecp6 of *C. fulvum* (Sánchez-Vallet et al., 2013) and Mg3LysM of *Mycosphaerella graminicola* (Marshall et al., 2011) also contain three LysM domains, multiple sequence alignment among LtScp1, Ecp6, and Mg3LysM was performed. The results showed that LtScp1 shares 51% and 43%

identity with Ecp6 and Mg3LysM in amino acid sequence, respectively (Figure S1D).

LtScp1 contributes to the full virulence of *L. theobromae*

To uncover the transcription profiles of *LtScp1* during the infection process, we performed qRT-PCR with cDNA reversely transcribed from RNA extracted from a susceptible grapevine cultivar (*Vitis vinifera* cv. 'Summer Black') infected with *L. theobromae*. Statistical analyses revealed that the transcript levels of *LtScp1* were obviously elevated at 12 h post inoculation (hpi), approximately 10-fold in comparison with that of vegetative hyphae, and reached its peak at 48 hpi (Figure 1A), suggesting that *LtScp1* was preferentially expressed at infectious stages.

To examine whether LtScp1 contributes to the virulence of *L. theobromae*, *LtScp1* gene was overexpressed and silenced in vivo via PEG-mediated protoplast transformation. Two transformants of each type were confirmed by qRT-PCR analyses and selected for pathogenicity analyses (Figure S2). Pathogenicity test on detached grapevine shoots showed that the overexpressed transformants LtScp1-OE1 and LtScp1-OE2 caused increased disease symptoms (Figure 1B), and the silenced transformants LtScp1-RNAi1 and LtScp1-RNAi2 caused attenuated disease symptoms when compared with that caused by wild type (Figure 1C).

To quantify the disease severity caused by the overexpressed transformants, wild type, and the silenced transformants, lesion lengths of diseased tissues were measured and analysed. Statistical analyses revealed that compared to wild type, the lesion lengths generated by the overexpressed transformants were higher (Figure 1D), and the lesion lengths generated by the silenced transformants were lower (Figure 1E). Collectively, pathogenicity tests indicate that LtScp1 is an important virulence factor in the symptom development caused by *L. theobromae*.

LtScp1 is a secreted protein

A genetic system based on the requirement of invertase secretion for yeast cells to grow on media with sucrose or raffinose as the sole carbon source has been widely used to identify secreted proteins (Fang et al., 2016; Gu et al., 2011; Jacobs et al., 1997; Tian et al., 2011). Here, LtScp1 was predicted to contain a signal peptide of 20 amino acids based on three different programmes (Figure 2A). To investigate the function of LtScp1 signal peptide, we engineered the fusion construct *pSUC2:LtScp1*, in which the LtScp1 signal peptide was located upstream of a truncated yeast invertase whose signal peptide had been removed. The signal peptide of Avr1b from *Phytophthora sojae* (*pSUC2:Avr1b*) and the first 25 amino acids of Mg87 from *Magnaporthe oryzae* (*pSUC2:Mg87*) were used as the positive and negative controls, respectively. Next, the fusion construct was transformed into the yeast strain YTK12, which was defective in invertase secretion. Similar to the positive control, only yeast transformants expressing *pSUC2:LtScp1* were capable of secreting invertase into extracellular spaces and grew on YPRAA media (with raffinose as the sole carbon source) and on CMD-W media (Figure 2B). Additionally, all transformed yeast cells grew on CMD-W media, and the yeast strain YTK12 did not grow on either YPRAA media or CMD-W media. The results suggest that the LtScp1 signal peptide functionally directs invertase to secretory pathway and that LtLtScp1 is a bona fide secreted protein.

LtScp1 is a *N*-glycosylated protein

N-Glycan is synthesized through the ordered assembly of a lipid-linked core structure $Glc_3Man_9GlcNAc_2$ in ER (Chen et al., 2014). To validate whether LtScp1 is a *N*-glycosylated protein, LtScp1 protein was transiently expressed in *N. benthamiana* and then subjected to treatment with endoglycosidase H (Endo H), a glycosidase that cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from *N*-linked glycoproteins, and PNGase F, an amidase that cleaves between the innermost GlcNAc and Asn residues of complex oligosaccharides from *N*-linked glycoproteins (Chen et al., 2014; Harishchandra et al., 2020). Subsequently, proteins digested by each enzyme were further investigated by immunoblotting analyses with an anti-GFP antibody. The results showed that proteins digested by both enzymes displayed smaller band sizes than the untreated LtScp1 protein (Figure 3A). To further confirm the modification, we mutated all five predicted *N*-glycosylation sites in LtScp1 simultaneously and then digested the mutated form LtScp1$^{N33/72/96/133/170G}$ with Endo H and PNGase F, followed by immunoblotting analyses. The results showed that the band size of the LtScp1$^{N33/72/96/133/170G}$ protein remained consistent before and after treatment with these two enzymes (Figure 3B).

LtScp1 was substantiated to be a *N*-glycosylated protein based on our results. To verify whether all putative *N*-glycosylation sites are indeed modified, we created five different point mutations in LtScp1 (LtScp1$^{N72/96/133/170G}$, LtScp1$^{N33/96/133/170G}$, LtScp1$^{N33/72/133/170G}$, LtScp1$^{N33/72/96/170G}$, and LtScp1$^{N33/72/96/133G}$). Next, each mutated form was treated with Endo H and PNGase F and then detected by immunoblotting tests. The results showed that three mutated forms, LtScp1$^{N33/96/133/170G}$, LtSc
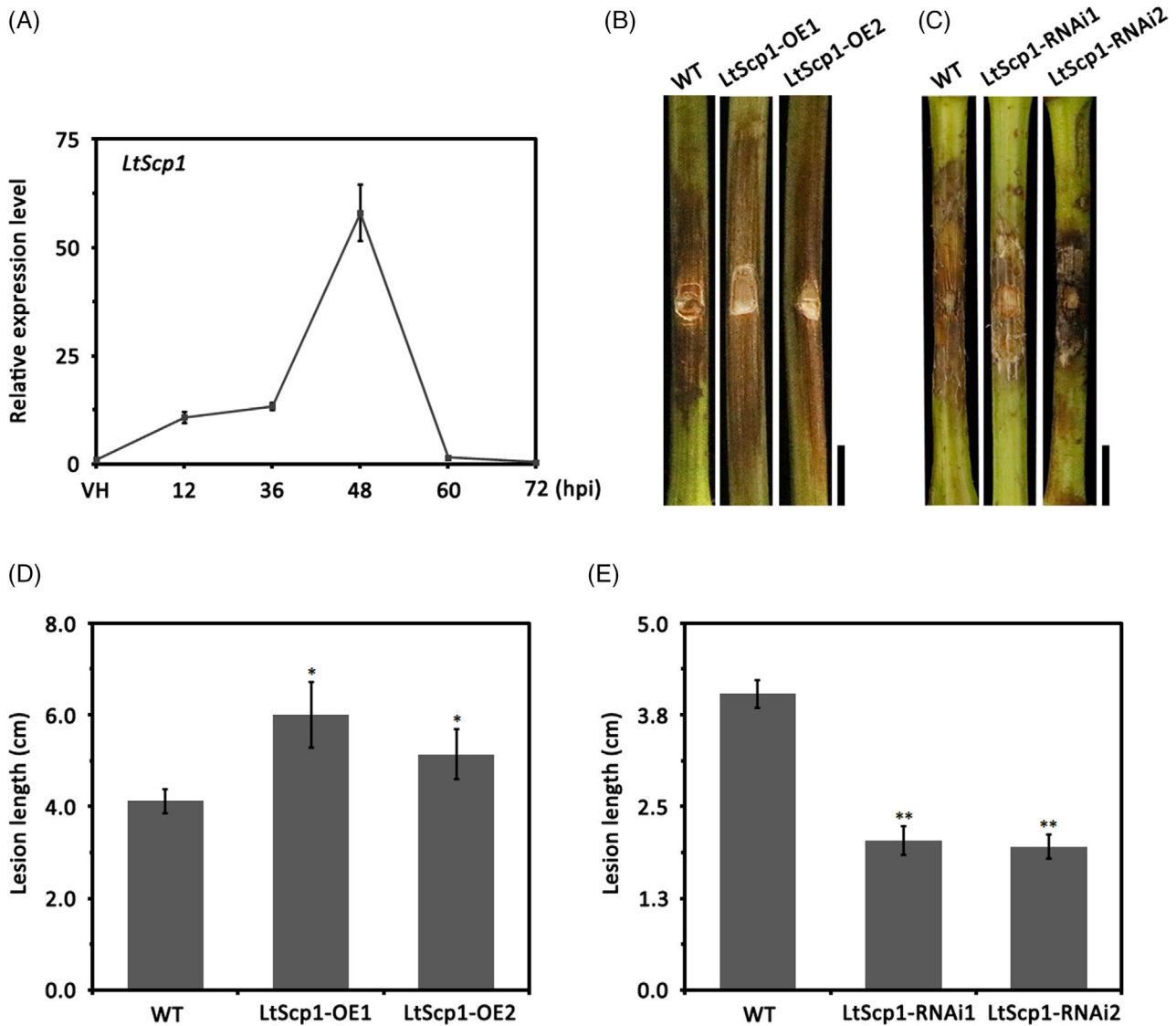
FIGURE 1 LtScp1 contributes to the virulence of *L. theobromae*. (A) Expression profiling of the *LtScp1* gene at infectious stages. The vegetative hyphae of wild-type cultured in complete medium and grapevine tissues infected by wild-type were harvested at 12, 36, 48, 60, and 72 h post inoculation (hpi) for RNA extraction. The isolated RNA was reversely transcribed into cDNA for *LtScp1* gene expression analyses via qRT-PCR assays. Expression data were normalized to the *actin* gene and calibrated against the transcript level of vegetative hyphae. The *actin* gene was used as the internal control. Relative transcript level of *LtScp1* gene was calculated with the $2^{-\Delta\Delta CT}$ method. Two biological repetitions with three replicates were assayed, and a representative dataset is presented. Data are means ± standard errors. VH, vegetative hyphae. (B) Pathogenicity test of the overexpressed transformants of *LtScp1*. One-year-old grapevine shoots were inoculated with mycelial plugs (5 mm in diameter) and then kept in a chamber under constant humidity and temperature. The inoculated grapevines were photographed at 3 days post inoculation (dpi). Bar = 1 cm. (C) Pathogenicity test of the silenced transformants of *LtScp1*. The inoculation experiment was performed using the same method described in (B). (D) Statistical analyses of lesion length caused by the overexpressed transformants of *LtScp1*. (E) Statistical analyses of lesion length caused by the silenced transformants of *LtScp1*. The means and standard errors were calculated from five replicates and the significant differences were indicated by stars. Significant differences were evaluated using the one-way analysis of variance (ANOVA) and least significant difference (LSD) tests ($**\alpha = 0.01$; $*\alpha = 0.05$).

1p1$^{N33/72/133/170G}$, and LtScp1$^{N33/72/96/170G}$, displayed larger band sizes than those digested by both enzymes (Figure 3C), indicating that Asn^{72}, Asn^{96}, and Asn^{133} were bona fide *N*-linked glycosylation sites in LtScp1. In contrast, another two mutation forms, LtScp1$^{N72/96/133/170G}$ and LtScp1$^{N33/72/96/133G}$, exhibited consistent band sizes before or after digestion by Endo

H and PNGase F (Figure 3D), suggesting that Asn^{33} and Asn^{170} were not *N*-glycosylated.

To provide more persuading evidences for above results, we further mutated the three *N*-glycosylation sites (Asn^{72}, Asn^{96}, and Asn^{133}) simultaneously, and the mutated form LtScp1$^{N72/96/133G}$ was treated with Endo H and PNGase F, followed by immunoblotting
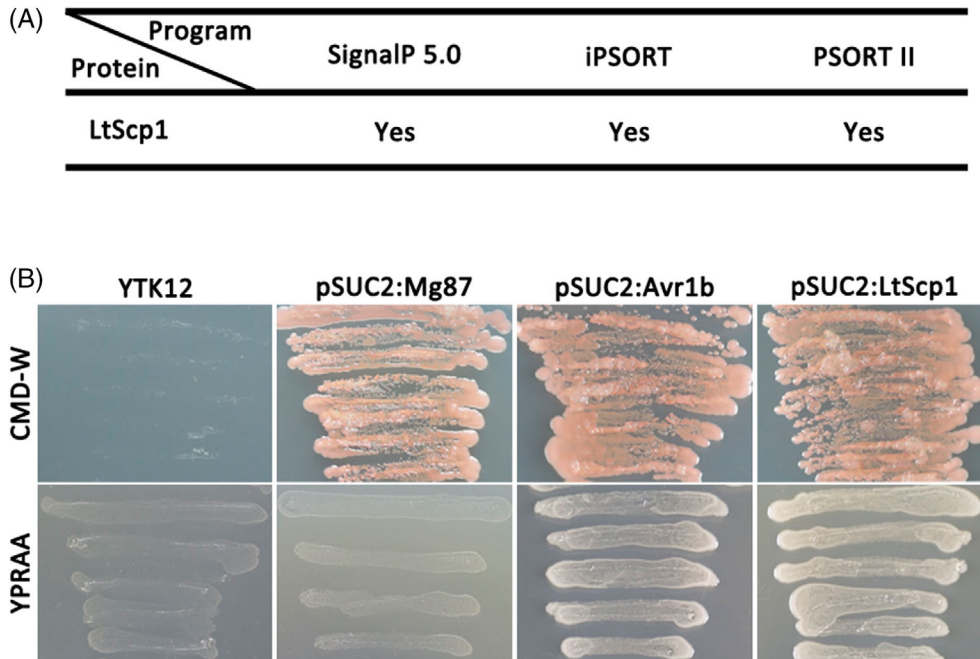
FIGURE 2 LtScp1 is a secreted protein. (A) Prediction of the LtScp1 signal peptide via SignalP 5.0, iPSORT, and PSORT II programmes. Positive predictions are labelled with yes. (B) Functional validation of the LtScp1 signal peptide using a yeast invertase secretion assay. The predicted coding sequence of the LtScp1 signal peptide was cloned into the *pSUC2* vector, in which the LtScp1 signal peptide was located upstream of a yeast invertase. The resultant fusion vector *pSUC2:LtScp1* was transformed into the yeast strain YTK12, which was defective in invertase secretion. Yeast cells expressing *pSUC2:LtScp1* grew on CMD-W and YPRAA (with raffinose as the sole carbon source) media. The yeast strain YTK12 did not grow on either CMD-W or YPRAA media. The N-terminal amino acid sequences of Avr1b from *P. sojae* and Mg87 from *M. oryzae* were used as positive and negative controls, respectively.
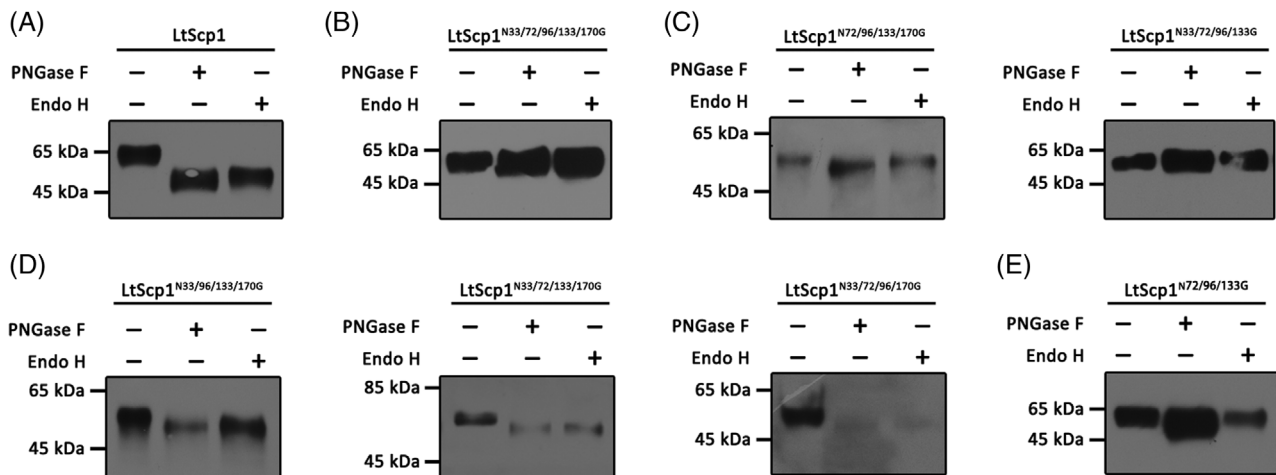

FIGURE 3 LtScp1 is an *N*-glycosylated protein. (A) Deglycosylation analysis of the LtScp1 protein. (B) Deglycosylation analysis of LtScp1 mutated form $LtScp1^{N33/72/96/133/170G}$. $LtScp1^{N33/72/96/133/170G}$ marks all five putative *N*-glycosylated amino acids Asn^{33}, Asn^{72}, Asn^{96}, Asn^{133}, and Asn^{170} in LtScp1 protein were mutated to amino acid Gly. (C) Deglycosylation analysis of LtScp1 mutated forms $LtScp1^{N72/96/133/170G}$ and $LtScp1^{N33/72/96/133G}$. (D) Deglycosylation analysis of LtScp1 mutated forms $LtScp1^{N33/96/133/170G}$, $LtScp1^{N33/72/133/170G}$ and $LtScp1^{N33/72/96/170G}$. (E) Deglycosylation analysis of LtScp1 mutated form $LtScp1^{N72/96/133/G}$. Total proteins isolated from *N. benthamiana* was subjected to treatment with endoglycosidase H (Endo H), a glycosidase that cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from *N*-linked glycoproteins, and PNGase F, an amidase that cleaves between the innermost GlcNAc and Asn residues of complex oligosaccharides from *N*-linked glycoproteins. Subsequently, proteins digested by each enzyme were further investigated by immunoblotting analyses with an anti-GFP antibody.

assays. The results showed that $LtScp1^{N72/96/133G}$ displayed consistent band sizes before and after treatment with Endo H and PNGase F enzymes (Figure 3E). Our results convincingly support that LtScp1 is indeed an *N*-glycosylated protein that contains three *N*-glycosylation sites.

The *N*-glycosylation of LtScp1 affects its dimerization

Many previously documented LysM proteins, including Ecp6 of *C. fulvum* (Sánchez-Vallet et al., 2013), Slp1 of *M. oryzae* (Mentlak et al., 2012), AtCERK1 of Arabidopsis (Liu, Li, et al., 2012; Liu, Liu, et al., 2012), OsCERK1 and CEBiP of rice (Shimizu et al., 2010) was able to form homo-/hetero-oligomers. In this study, we tested to determine whether LtScp1 shared similar molecular features. Therefore, we used the yeast two-hybrid system to examine the interaction between LtScp1 molecules. The results showed that strong interaction between LtScp1 monomer was detected (Figure 4A), suggesting that LtScp1 monomers had the capacity to form dimers.

To determine whether the *N*-glycosylation of LtScp1 affected its dimerization, we mutated the three *N*-glycosylation sites (N^{72}, N^{96}, and N^{133}) individually or simultaneously, and engineered a total of seven mutated forms of LtScp1 (LtScp1^{N72G}, LtScp1^{N96G}, LtScp1^{N133G}, LtScp1$^{N72/96}$, LtScp1$^{N72/133G}$, LtScp1$^{N96/133G}$, and LtScp1$^{N72/96/133G}$). Next, interactions among these mutants were detected using the yeast two-hybrid assays. The results showed that molecular interactions between partial mutant pairs

including LtScp1$^{N72/96G}$/LtScp1$^{N72/96/133G}$, LtScp1$^{N72/133G}$/LtScp1$^{N72/96/133G}$, LtScp1$^{N96/133G}$/LtScp1$^{N72/96/133G}$, and LtScp1$^{N72/96/133G}$/LtScp1$^{N72/96/133G}$ were attenuated or even disappeared (Figure 4B,C), indicating that *N*-glycosylation of LtScp1 influenced its dimerization.

LtScp1 associates with grapevine chitinase VvChi4

To further explore the functional mechanism by which LtScp1 contributed to the virulence of *L. theobromae*, we performed a yeast two-hybrid screening with LtScp1$^{\Delta SP}$ as the bait against the cDNA library constructed with RNA extracted from grapevine. A total of seven candidates, including a putative class IV chitinase, named VvChi4, were identified to potentially interact with LtScp1 (Table S1).

Afterwards, the interactions between LtScp1 and VvChi4 were further examined by high-stringency yeast two-hybrid assays. We initially engineered *pGBKT7*: *LtScp1*$^{\Delta SP}$ bait construct and *pGADT7:VvChi4*$^{\Delta SP}$ prey construct and then transformed both vectors into yeast strain AH109 simultaneously. The resulting yeast transformants were tested for their growth on synthetic
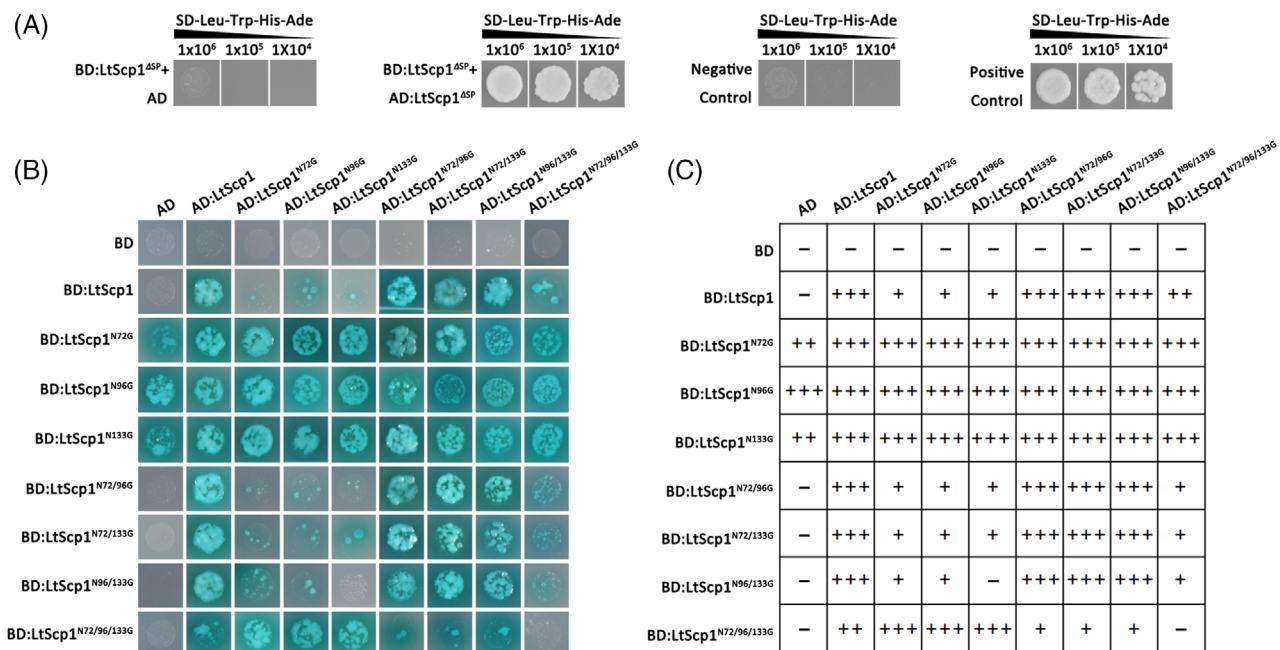

FIGURE 4 *N*-glycosylation of LtScp1 affects its dimerization. (A) Self-interaction analyses of the LtScp1 molecule by yeast two-hybrid assays. The *LtScp1*$^{\Delta SP}$ cDNA was cloned into the bait vector *pGBKT7* and the prey vector *pGADT7* simultaneously. Subsequently, both fusion constructs were cotransformed into the yeast strain AH109 using the PEG/LiAc method. Yeast cells carrying LtScp1$^{\Delta SP}$ bait vector (*BD: LtScp1*$^{\Delta SP}$) and LtScp1$^{\Delta SP}$ prey vector (*AD:LtScp1*$^{\Delta SP}$) were tested for their growth on synthetic dropout media (SD-Leu-Trp-His-Ade). The interaction between empty vector *pGADT7* with *pGBKT7* was used as a negative control. The interaction between *pGADT7-T* with *pGBKT7-53* was used as a positive control. $^{\Delta SP}$, Protein without its signal peptide. (B) Self-interaction analyses of LtScp1 point mutants. LtScp1$^{N72/96/133G}$ marks the putative *N*-glycosylation sites Asn^{72}, Asn^{96}, and Asn^{133} in LtScp1 protein were mutated to amino acid Gly. Yeast cells carrying the bait and prey vectors were tested for their growth on SD-Leu-Trp-His-Ade media and for *β*-galactosidase (LacZ) activities. (C) Interaction intensity was grouped and marked with different symbols. +++, strong level. ++, moderate level. +, weak level. −, no interaction

dropout media and β-galactosidase activity. The results revealed that yeast cells expressing both constructs grew on SD-Leu-Trp-His media and displayed strong β-galactosidase activity (Figure 5A), suggesting that LtScp1 interacted with VvChi4.

To map the key regions of VvChi4 that interact with LtScp1, a series of truncated forms of VvChi4 were coexpressed with LtScp1 in yeast. Yeast transformants expressing the Glyco_hydro_19 domain of VvChi4 (amino acid residues 55–216) with LtScp1 grew on SD-
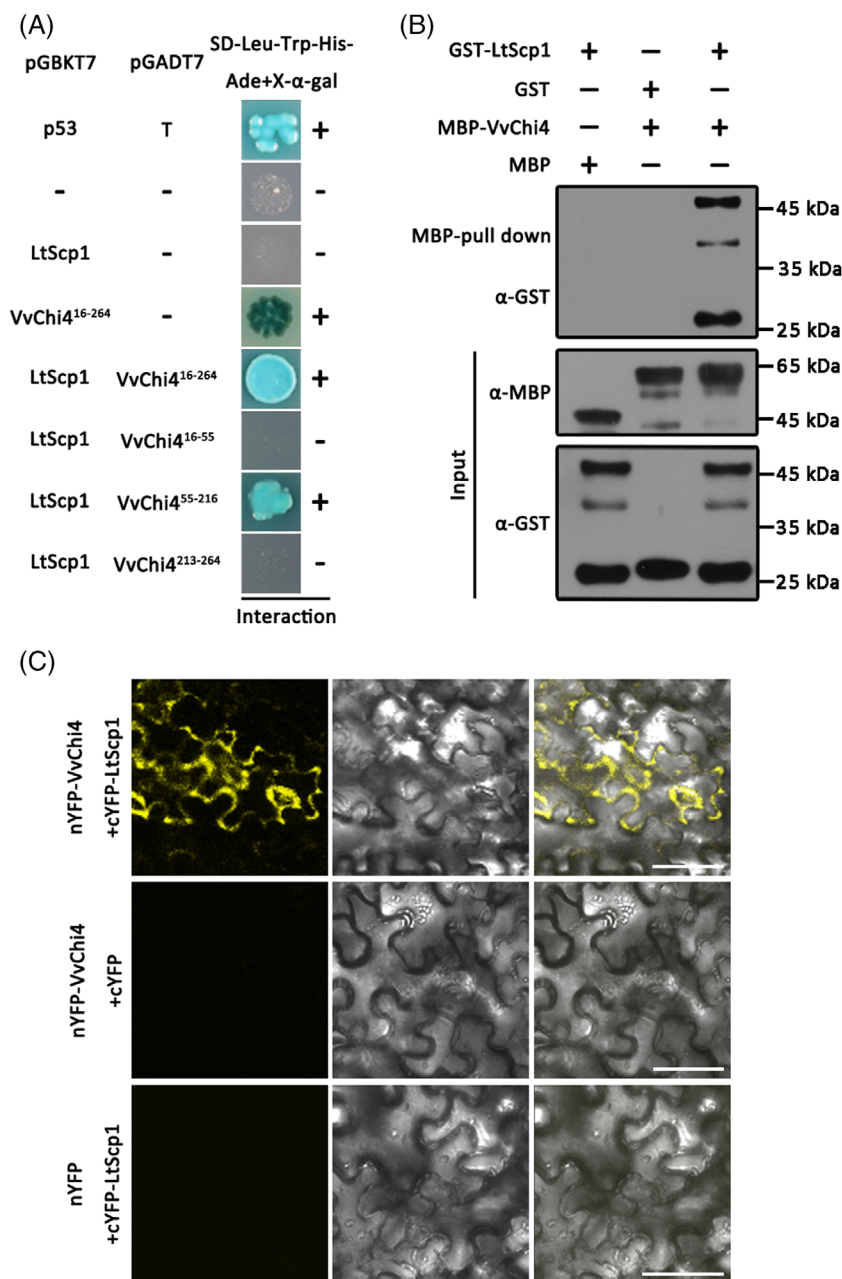

FIGURE 5 *L. theobromae* protein LtScp1 interacts with grapevine chitinase VvChi4. (A) Interaction analyses of LtScp1 with VvChi4 or with a series of truncated forms of VvChi4. The *LtScp1*$^{\Delta SP}$ ORF was cloned into the bait vector *pGBKT7*, and the *VvChi4*$^{\Delta SP}$ ORF with its truncated forms were cloned into the prey vector *pGADT7*. Subsequently, the bait and prey fusion constructs were cotransformed into the yeast strain AH109 using the PEG/LiAc method. Truncated moiety, for example, VvChi4$^{16-55}$, indicates protein fragment ranging from amino acid positions 16 to 55. Yeast cells carrying the bait and prey vectors were tested for their growth on SD-Leu-Trp-His-Ade media and for β-galactosidase (LacZ) activity. The interaction between empty vector *pGADT7* with *pGBKT7* was used as a negative control. The interaction between *pGADT7-T* with *pGBKT7-53* was used as a positive control. (B) Interaction analyses of LtScp1 with VvChi4 via MBP pull down experiments. Recombinant proteins MBP-VvChi4 and GST-LtScp1 were subjected to MBP pull down analyses. Interactions between LtScp1 with VvChi4 were examined by immunoblotting assays. (C) Interaction analyses of LtScp1 with VvChi4 via split YFP assays. The *A. tumefaciens* expressing *p35S:nYFP-VvChi4* and *p35S:cYFP-LtScp1* were coinfiltrated into 4-week-old *N. benthamiana* leaves. The images were taken using a confocal laser scanning microscope at 2 days post infiltration (dpi). Bar = 50 μm

Leu-Trp-His media and displayed strong β-galactosidase activity (Figure 5A), suggesting that the Glyco_hydro_19 domain of VvChi4 was the key region that interacted with LtScp1.

To supply more cogent evidence about the interaction between LtScp1 and VvChi4, further MBP pull-down experiments were conducted. The GST-LtScp1 protein purified from *Escherichia coli* using GST beads was incubated with MBP-VvChi4 protein purified from *E. coli* BL21 using MBP magnetic beads. Protein immunoblotting analyses showed that LtScp1 was pulled down by the VvChi4 protein (Figure 5B). Moreover, we tested the interaction between LtScp1 and VvChi4 *in planta*. LtScp1 was confirmed to interact with VvChi4 in *N. benthamiana* leaves via BiFC assays (Figure 5C). Altogether, these results strongly supported the physical interaction of LtScp1 with VvChi4.

LtScp1 interferes the ability of VvChi4 to bind chitin

Based on amino acid sequence analyses using SignalP 5.0 and Pfam programme, VvChi4 was predicted to contain one N-terminal signal peptide with 20 amino acids, one chitin-binding domain, and two glycolysis hydrolysis domains (Figure 6A). In previous research, two chitinases from *Vitis vinifera* L. cv 'Shiraz' have been identified (Robinson et al., 1997). Based on amino acid sequence alignment, VvChi4 identified in *Vitis vinifera* L. cv 'Shiraz' shares over 95% identity with VvChi4 identified in *Vitis vinifera* L. cv 'Summer Black' (Figure 6B), implying that these genes are most likely homologues from different grapevine species.

To investigate the function of the VvChi4 signal peptide, we engineered the fusion construct *pSUC2: VvChi4*, in which the LtScp1 signal peptide was located upstream of a truncated yeast invertase, whose signal peptide had been removed. Yeast expressing *pSUC2:Avr1b* and *pSUC2:Mg87* were used as the positive and negative controls, respectively. Next, the fusion construct was transformed into the yeast strain YTK12, which was defective in invertase secretion. Same to the positive control, yeast transformants expressing *pSUC2:VvChi4* were capable of secreting invertase into extracellular spaces and grew on YPRAA media and on CMD-W media (Figure 6C). Additionally, all transformed yeast cells grew on CMD-W media, and the yeast strain YTK12 did not grown on either YPRAA media or CMD-W media. The results suggest that the VvChi4 signal peptide functionally directs invertase to the secretory pathway and that VvChi4 is indeed a secreted protein, which supports that LtScp1 interacts with VvChi4 in the apoplastic space.

As VvChi4 interacted with LtScp1, we tried to examine the transcription profile of VvChi4 during the infection. Therefore, we performed qRT-PCR with cDNA transcribed from RNA extracted from a susceptible grapevine cultivar (*Vitis vinifera* L. 'Summer Black') infected by *L. theobromae*. Statistical analysis revealed that the transcript level of VvChi4 was significantly elevated at 12 and 60 hpi, approximately 7-fold in comparison with that at 0 hpi (Figure 6D), suggesting that *VvChi4* was preferentially transcribed at the early and late infectious stages.

Because of the interaction between VvChi4 and LtScp1, we tested to investigate whether LtScp1 interferes with the ability of VvChi4 to bind chitin directly. Therefore, we performed a chitin competitive binding assay in which VvChi4 and LtScp1 proteins were incubated with chitin beads simultaneously. When the content of LtScp1 protein was gradually increased, binding of chitin beads to VvChi4 protein was gradually decreased (Figure 6E), suggesting that LtScp1 protein competes for chitin binding with grapevine chitinase VvChi4.

Moreover, the chitinase activity of VvChi4 (from *Vitis vinifera* L. cv 'Shiraz') with chitin as the substrate has been reported (Robinson et al., 1997). Based on these results, we proposed a working model in which LtScp1 protects fungal mycelium from degradation by sheltering chitin from being recognized by VvChi4 and avoids the immune response triggered by chitin during the infection process (Figure 7).

DISCUSSION

LysM proteins have been widely characterized in many herbaceous plant pathogens (Buist et al., 2008; Kombrink et al., 2011), but they are rarely reported in the woody plant pathogen *L. theobromae*, which has been reported to cause considerable economic losses in the agricultural industry annually (Úrbez-Torres et al., 2008; Yan et al., 2013, 2018). In this study, we investigated the mechanism by which *L. theobromae* successfully colonizes grapevine tissues and mainly focused on whether and how the LysM proteins secreted by *L. theobromae* were associated with fungal pathogenicity.

The LysM domain has been reported to be essential for the biological function of LysM proteins. In the current study, LtScp1 was predicted to contain three LysM domains, similar to Ecp6 and Mg3LysM. Structural analyses of Ecp6 showed that the composite LysM1-LysM3 binding site provides a single binding event with ultrahigh (pM) affinity for chitin binding to sequester chitin oligosaccharides. The LysM2 domain, in contrast, may have a secondary working mechanism in which the LysM2 domain perturbs the activation of chitin-triggered immunity by preventing the immune receptor dimerization that is required for the activation of immune signalling (Liu, Li, et al., 2012; Liu, Liu, et al., 2012; Sánchez-Vallet et al., 2013). Sequence
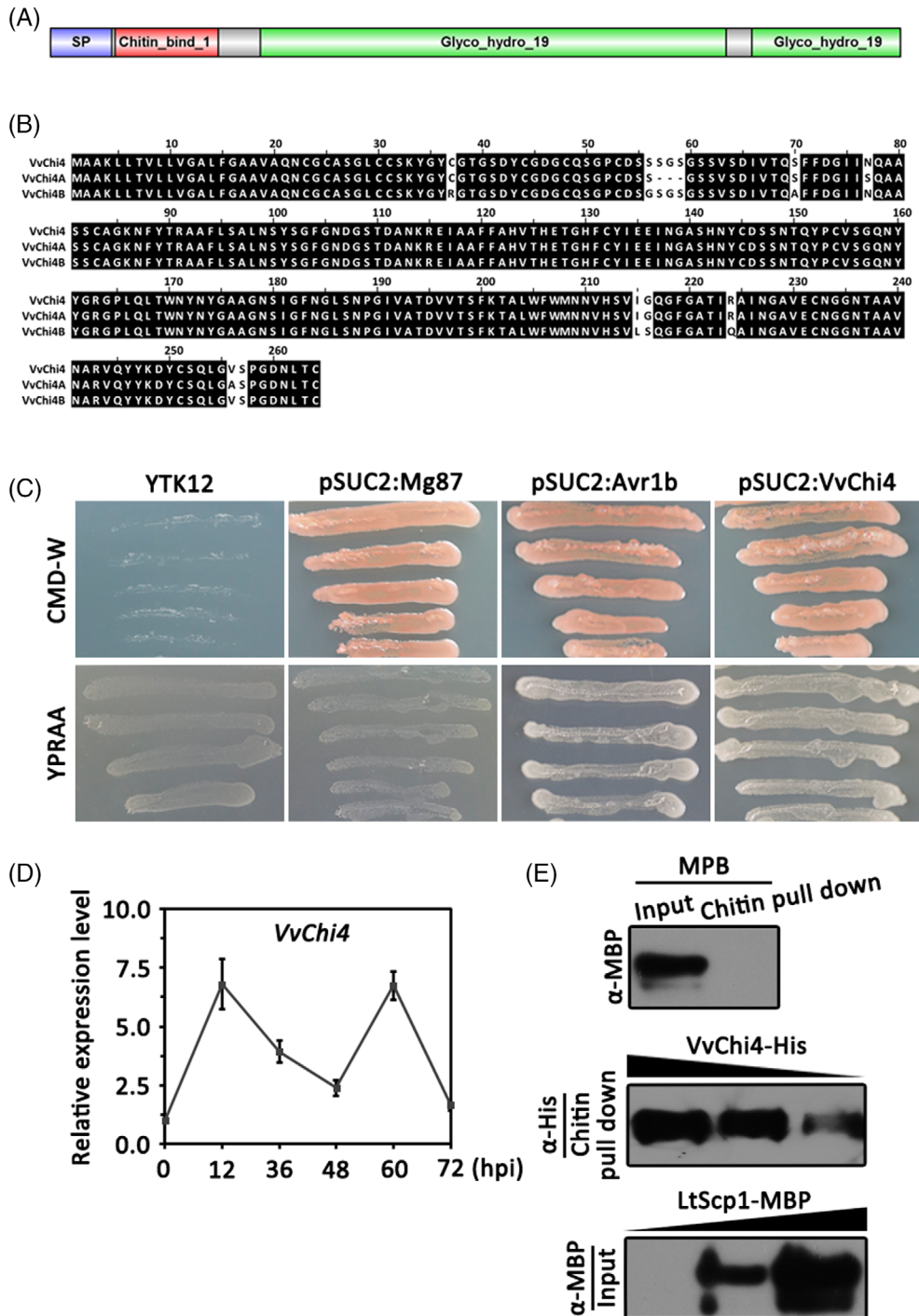
(A)

(B)

(C)

(D)

(E)

FIGURE 6 LtScp1 interferes the ability of VvChi4 to bind chitin. (A) Schematic illustration of the VvChi4 protein. The blue box indicates a predicted signal peptide. The red box signifies a putative chitin-binding domain. The green box marks a predicted glycolysis hydrolysis domain. (B) Amino acid sequence alignments of VvChi4 homologues from different grapevine species. The sequences of all three proteins were aligned with ClustalX2, and the alignments were edited with Jalview. Identical amino acids were highlighted with black boxes. (C) Functional validation of the VvChi4 signal peptide using a yeast invertase secretion assay. The predicted coding sequence of the VvChi4 signal peptide was cloned into the *pSUC2* vector, in which the VvChi4 signal peptide was located upstream of a yeast invertase. The resultant fusion vector *pSUC2:VvChi4* was transformed into the yeast strain YTK12, which was defective in invertase secretion. Yeast cells expressing *pSUC2:VvChi4* grew on CMD-W and YPRAA media. Yeast strain YTK12 did not grow on either CMD-W or YPRAA media. The N-terminal amino acid sequences of Avr1b from *P. sojae* and Mg87 from *M. oryzae* were used as positive and negative controls, respectively. (D) Expression profiling of the *VvChi4* gene during the infection process. The grapevine shoots infected by *L. theobromae* were harvested at 0, 12, 36, 48, 60, and 72 h post inoculation (hpi) for *VvChi4* expression analyses via qRT-PCR. The *actin* gene was used as the internal control. Expression data were normalized to the *actin* gene and calibrated against the transcript level at 0 hpi. The relative transcript level of the *VvChi4* gene was calculated with the $2^{-\Delta\Delta CT}$ method. Two biological repetitions with three replicates were assayed, and a representative dataset is presented. Data are means $\pm$ standard errors. (E) Competitive binding of LtScp1 and VvChi4 proteins to chitin beads. The VvChi4-His and LtScp1-MBP proteins were expressed in *Pichia pastoris* and then incubated with insoluble chitin beads at 4°C on a gentle rocking platform, followed by immunoblotting assays. Each 200-μl reaction mixture comprised 50 μl chitin beads and 30 μg VvChi4-His protein. A gradually increased content of LtScp1-MBP protein (0, 20, and 50 μg) was added into the reaction mixture.
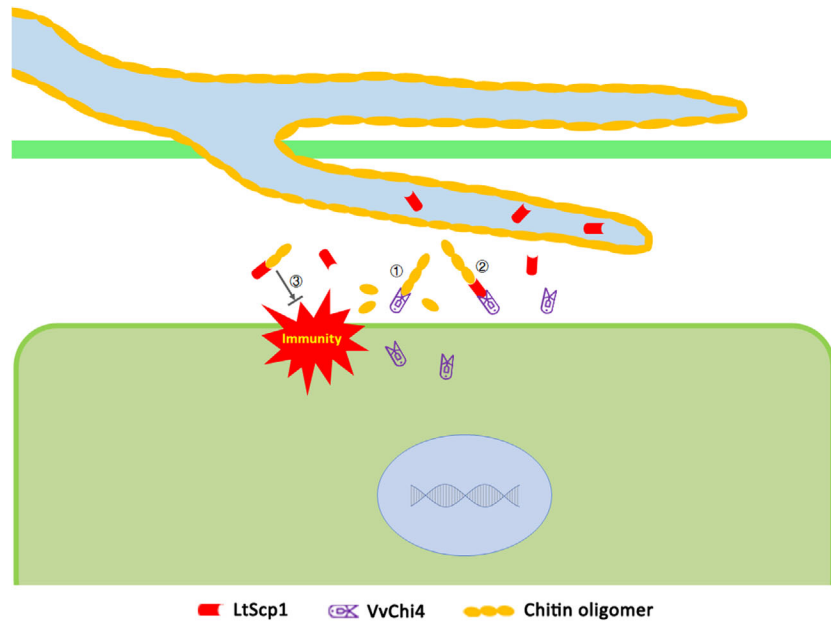
FIGURE 7 Putative working model of LtScp1. ①, VvChi4 binds to chitin and degrades the chitin oligomer. ②, LtScp1 interacts with VvChi4 to protect chitin oligomers against hydrolysis by VvChi4. ③, LtScp1 sequester chitin oligosaccharides to avoid plant immunity triggered by chitin

alignments showed that LtScp1 shared high sequence similarities with Ecp6 and Mg3LysM, but it is unknown whether the LysM domains in LtScp1 function in a similar manner as those in Ecp6 and Mg3LysM. Moreover, LtScp1 molecule was able to interact with itself and this feature was shared by another reported LysM protein Slp1 (Mentlak et al., 2012). Upon these results, it is assumed that LtScp1 molecules may also form a multimeric complex and perform their biological function through the collective action of multiple LysM domains. Intensive research on the LtScp1 protein structure in vivo will enhance our understanding of the biochemical function of LysM domains in *L. theobromae*.

N-glycosylation modification is necessary for the maturation of newly synthesized proteins. Here, three *N*-glycosylation sites in LtScp1 were identified via point mutation analyses. Interestingly, when the *N*-glycosylation site Asn[133] was not mutated in LtScp1, the band intensity of the protein LtScp1[N33/72/96/170G] was significantly decreased after digestion with PNGase F and Endo H, suggesting that *N*-glycosylation of Asn[133] may affect the protein stability of LtScp1. Moreover, it was found that *N*-glycosylation regulated the dimerization of LtScp1 molecules, whereas whether the dimerization affected its biological function was unrevealed in the current study, because it was technically difficult to operate target genes in this multinuclear fungus. Further structural analyses may be conducive to answer this question.

Chitinases, a group of degrading enzymes with chitin as the enzymatic substrate, were generally regarded as pathogenesis-related proteins and implicated in

plant defence against pathogen infection (Kombrink et al., 1988; Margis-Pinheiro et al., 1993; Naumann & Price, 2012; Salzer et al., 2004). In the current study, the grapevine protein VvChi4, a secreted chitinase, was confirmed to interact with LtScp1, which provided an additional piece of evidence to support the putative apoplastic localization of LtScp1 as LysM effectors were assumed to play roles in the intercellular spaces. Moreover, the chitinase activities of VvChi4A and VvChi4B in grape berries were reported by Robinson et al. (1997) and these proteins share over 95% amino acid identity with VvChi4 identified in this study. Additionally, plant chitinases were found to be capable of inhibiting fungal hyphal growth and mediating plant defence responses (Huynh et al., 1992; Kaku et al., 2006; Naumann & Price, 2012; Schlumbaum et al., 1986). Furthermore, LtScp1 was confirmed to be a competitive inhibitor of VvChi4 in our research. Based on our results and previous studies, it can be assumed that LtScp1 protects fungal mycelium from degradation by sheltering chitin from being recognized by VvChi4 and avoids the immune response triggered by chitin during the infection process.

In summary, characterizations of previously documented LysM effectors have mainly focused on the suppression of chitin-induced immune responses and chitin binding abilities. In contrast, interacting targets of host plants have received relatively less attention. Here, we functionally characterize a LysM protein LtScp1 in *L. theobromae* and propose a working model to reveal the regulatory mechanism of LtScp1 in the apoplastic space.

AUTHOR CONTRIBUTIONS

Jiye Yan and Junbo Peng conceived and designed the experiments. Junbo Peng and Jiye Yan performed the experiments. Jiye Yan and Junbo Peng analysed the data. Jiye Yan and Junbo Peng wrote and revised the manuscript. Wei Zhang helped to revise the manuscript. Xinghong Li, Yonghua Li, and Ying Zhou assisted and advised during the molecular experiments and pathogenicity tests.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary materials of this article.

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REFERENCES

Akcapinar, G.B., Kappel, L., Sezerman, O.U. & Seidl-Seiboth, V. (2015) Molecular diversity of LysM carbohydrate-binding motifs in fungi. *Current Genetics*, 61, 103–113.

An, G., Ebert, P.R., Mitra, A. & Ha, S.B. (1988) Binary vectors. In: Gelvin, S.B. & Schilperoort, R.A. (Eds.) *Plant molecular biology manual*. Dordrecht, Netherlands: Kluwer Academic Publishers, pp. 1–19.

Boller, T. & He, S.Y. (2009) Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science*, 324, 742–744.

Bolton, M.D., van Esse, H.P., Vossen, J.H., de Jonge, R., Stergiopoulos, I., Stulemeijer, I.J.E. et al. (2008) The novel *Cladosporium fulvum* lysin motif effector Ecp6 is a virulence factor with orthologues in other fungal species. *Molecular Microbiology*, 69, 119–136.

Buist, G., Steen, A., Kok, J. & Kuipers, O.P. (2008) LysM, a widely distributed protein motif for binding to (peptido) glycans. *Molecular Microbiology*, 68, 838–847.

Carlucci, A., Cibelli, F., Lops, F. & Raimondo, M.L. (2015) Characterization of Botryosphaeriaceae species as causal agents of trunk diseases on grapevines. *Plant Disease*, 99, 1678–1688.

Chen, X.L., Shi, T., Yang, J., Shi, W., Gao, X., Chen, D. et al. (2014) *N*-glycosylation of effector proteins by an α-1,3-mannosyltransferase is required for the rice blast fungus to evade host innate immunity. *The Plant Cell*, 26, 1360–1376.

Chethana, K.W.T., Li, X., Zhang, W., Hyde, K.D. & Yan, J. (2016) Trail of decryption of molecular research on Botryosphaeriaceae in woody plants. *Phytopathologia Mediterranea*, 55, 147–171.

Couto, D. & Zipfel, C. (2016) Regulation of pattern recognition receptor signalling in plants. *Nature Reviews Immunology*, 16, 537–552.

Davis, E.L., Hussey, R.S., Mitchum, M.G. & Baum, T.J. (2008) Parasitism proteins in nematode-plant interactions. *Current Opinion in Plant Biology*, 11, 360–366.

de Jonge, R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R. et al. (2010) Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science*, 329, 953–955.

de Wit, P.J. (2007) How plants recognize pathogens and defend themselves. *Cellular and Molecular Life Sciences*, 64, 2726–2732.

El-Goorani, M.A., El-Meleigi, M.A., Elarosi, H.M. & Wasfy, E.H. (1972) Dieback of grapevine by *Botryodiplodia theobromae* Pat. in Egypt. *Phytopathologia Mediterranea*, 11, 210–211.

Fang, A., Han, Y., Zhang, N., Zhang, M., Liu, L., Li, S. et al. (2016) Identification and characterization of plant cell death-inducing secreted proteins from *Ustilaginoidea virens*. *Molecular Plant-Microbe Interactions*, 29, 405–416.

Galán, J.E. & Collmer, A. (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science*, 284, 1322–1328.

Galán, J.E. & Wolf-Watz, H. (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature*, 444, 567–573.

Garvey, K.J., Saedi, M.S. & Ito, J. (1986) Nucleotide sequence of Bacillus phage Φ29 genes 14 and 15: homology of gene 15 with other phage lysozymes. *Nucleic Acids Research*, 14, 10001–10008.

Gu, B., Kale, S.D., Wang, Q., Pan, Q., Cao, H., Meng, Y. et al. (2011) Rust secreted protein Ps87 is conserved in diverse fungal pathogens and contains a RXLR-like motif sufficient for translocation into plant cells. *PLoS One*, 6, e27217.

Han, X. & Kahmann, R. (2019) Manipulation of phytohormone pathways by effectors of filamentous plant pathogens. *Frontiers in Plant Science*, 10, 822.

Harishchandra, D.L., Zhang, W., Li, X., Chethana, K.W.T., Hyde, K.D., Brooks, S. et al. (2020) A LysM domain-containing protein LtLysM1 is important for vegetative growth and pathogenesis in woody plant pathogen *Lasiodiplodia theobromae*. *The Plant Pathology Journal*, 36, 323–334.

Hogenhout, S.A., Van Der Hoorn, R.A.L., Terauchi, R. & Kamoun, S. (2009) Emerging concepts in effector biology of plant-associated organisms. *Molecular Plant-Microbe Interactions*, 22, 115–122.

Huynh, Q.K., Hironaka, C.M., Levine, E.B., Smith, C.E., Borgmeyer, J.R. & Shah, D.M. (1992) Antifungal proteins from plants. Purification, molecular cloning, and antifungal properties of chitinases from maize seed. *Journal of Biological Chemistry*, 267, 6635–6640.

Jacobs, K.A., Collins-Racie, L.A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K. et al. (1997) A genetic selection for isolating cDNAs encoding secreted proteins. *Gene*, 198, 289–296.

Jones, J.D.G. & Dangl, J.L. (2006) The plant immune system. *Nature*, 444, 323–329.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K. et al. (2006) Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences USA*, 103, 11086–11091.

Kombrink, A., Rovenich, H., Shi-Kunne, X., Rojas-Pandilla, E., van den Berg, G.C.M., Domazakis, E. et al. (2017) *Verticillium dahliae* LysM effectors differentially contribute to virulence on plant hosts. *Molecular Plant Pathology*, 18, 596–608.

Kombrink, A., Sánchez-Vallet, A. & Thomma, B.P. (2011) The role of chitin detection in plant-pathogen interactions. *Microbes and Infection*, 13, 1168–1176.

Kombrink, E., Schröder, M. & Hahlbrock, K. (1988) Several "pathogenesis-related" proteins in potato are 1, 3-β-glucanases and chitinases. *Proceedings of the National Academy of Sciences USA*, 85, 782–786.

Liu, B., Li, J.F., Ao, Y., Qu, J., Li, Z., Su, J. et al. (2012) Lysin motif-containing proteins LYP4 and LYP6 play dual roles in

peptidoglycan and chitin perception in rice innate immunity. *The Plant Cell*, 24, 3406–3419.

Liu, L., Xu, L., Jia, Q., Pan, R., Oelmüller, R., Zhang, W. et al. (2019) Arms race: diverse effector proteins with conserved motifs. *Plant Signaling & Behavior*, 14, e1557008.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J. et al. (2012) Chitin-induced dimerization activates a plant immune receptor. *Science*, 336, 1160–1164.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25, 402–408.

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M. et al. (2015) Fungal effectors and plant susceptibility. *Annual Review of Plant Biology*, 66, 513–545.

Lombard, V., Ramulu, H.G., Drula, E., Coutinho, P.M. & Henrissat, B. (2014) The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Research*, 42, D490–D495.

Macho, A.P. & Zipfel, C. (2014) Plant PRRs and the activation of innate immune signaling. *Molecular Cell*, 54, 263–272.

Mafurah, J.J., Ma, H., Zhang, M.X., Xu, J., He, F., Ye, T.Y. et al. (2015) A virulence essential CRN effector of *Phytophthora capsici* suppresses host defense and induces cell death in plant nucleus. *PLoS One*, 10, e0127965.

Margis-Pinheiro, M., Martin, C., Didierjean, L. & Burkard, G. (1993) Differential expression of bean chitinase genes by virus infection, chemical treatment and UV irradiation. *Plant Molecular Biology*, 22, 659–668.

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K.E. et al. (2011) Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology*, 156, 756–769.

Mentlak, T.A., Kombrink, A., Shinya, T., Ryder, L.S., Otomo, I., Saitoh, H. et al. (2012) Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. *The Plant Cell*, 24, 322–335.

Naumann, T.A. & Price, N.P. (2012) Truncation of class IV chitinases from Arabidopsis by secreted fungal proteases. *Molecular Plant Pathology*, 13, 1135–1139.

Newman, M.A., Sundelin, T., Nielsen, J.T. & Erbs, G. (2013) MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Frontiers in Plant Science*, 4, 139.

Oh, S.K., Young, C., Lee, M., Oliva, R., Bozkurt, T.O., Cano, L.M. et al. (2009) In planta expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes, including activation of the *Solanum bulbocastanum* disease resistance protein Rpi-blb2. *The Plant Cell*, 21, 2928–2947.

Reis, P., Pierron, R., Larignon, P., Lecomte, P., Abou-Mansour, E., Farine, S. et al. (2019) Vitis methods to understand and develop strategies for diagnosis and sustainable control of grapevine trunk diseases. *Phytopathology*, 109, 916–931.

Robinson, S.P., Jacobs, A.K. & Dry, I.B. (1997) A class IV chitinase is highly expressed in grape berries during ripening. *Plant Physiology*, 114, 771–778.

Salzer, P., Feddermann, N., Wiemken, A., Boller, T. & Staehelin, C. (2004) Sinorhizobium meliloti-induced chitinase gene expression in *Medicago truncatula* ecotype R108-1: a comparison between symbiosis-specific class V and defence-related class IV chitinases. *Planta*, 219, 626–638.

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D.J., Thomma, B.P.H.J. et al. (2013) Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *eLife*, 2, e00790.

Schlumbaum, A., Mauch, F., Vögeli, U. & Boller, T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature*, 324, 365–367.

Selin, C., de Kievit, T.R., Belmonte, M.F. & Fernando, W.G.D. (2016) Elucidating the role of effectors in plant-fungal interactions: progress and challenges. *Frontiers in Microbiology.*, 7, 600.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y. et al. (2010) Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *The Plant Journal*, 64, 204–214.

Slippers, B. & Wingfield, M.J. (2007) Botryosphaeriaceaeas endophytes and latent pathogens of woody plants: diversity, ecology andimpact. *Fungal Biology Reviews*, 21, 90–106.

Songy, A., Fernandez, O., Clément, C., Larignon, P. & Fontaine, F. (2019) Grapevine trunk diseases under thermal and water stresses. *Planta*, 249, 1655–1679. https://doi.org/10.1007/s00425-019-03111-8

Stergiopoulos, I. & de Wit, P.J. (2009) Fungal effector proteins. *Annual Review of Phytopathology*, 47, 233–263.

Takahara, H., Hacquard, S., Kombrink, A., Hughes, H.B., Halder, V., Robin, G.P. et al. (2016) *Colletotrichum higginsianum* extracellular LysM proteins play dual roles in appressorial function and suppression of chitin-triggered plant immunity. *New Phytologist*, 211, 1323–1327.

Thomma, B.P.H.J., Nürnberger, T. & Joosten, M.H. (2011) Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *The Plant Cell*, 23, 4–15.

Tian, H., Mackenzie, C.I., Rodriguez-Moreno, L., van den Berg, G.C.M., Chen, H., Rudd, J.J. et al. (2021) Three LysM effectors of *Zymoseptoria tritici* collectively disarm chitin-triggered plant immunity. *Molecular Plant Pathology*, 22, 683–693.

Tian, M.Y., Win, J., Savory, E., Burkhardt, A., Held, M., Brandizzi, F. et al. (2011) 454 genome sequencing of *Pseudoperonospora cubensis* reveals effector proteins with a putative QXLR translocation motif. *Molecular Plant-Microbe Interactions*, 24, 543–553.

Úrbez-Torres, J.R., Leavitt, G.M., Guerrero, J.C., Guevara, J. & Gubler, W.D. (2008) Identification and pathogenicity of *Lasiodiplodia theobromae* and *Diplodia seriata*, the causal agents of bot canker disease of grapevines in Mexico. *Plant Disease*, 92, 519–529.

Wang, X., Jiang, N., Liu, J., Liu, W. & Wang, G.L. (2014) The role of effectors and host immunity in plant-necrotrophic fungal interactions. *Virulence*, 5, 722–732.

Win, J., Chaparro-Garcia, A., Belhaj, K., Saunders, D.G.O., Yoshida, K., Dong, S. et al. (2012) Effector biology of plant-associated organisms: concepts and perspectives. *Cold Spring Harbor Symposia on Quantitative Biology*, 77, 235–247.

Yan, J.Y., Xie, Y., Zhang, W., Wang, Y., Liu, J.K., Hyde, K.D. et al. (2013) Species of *Botryosphaeriaceae* involved in grapevine dieback in China. *Fungal Diversity*, 61, 221–236.

Yan, J.Y., Zhao, W.S., Chen, Z., Xing, Q.K., Zhang, W., Chethana, K.W.T. et al. (2018) Comparative genome and transcriptome analyses reveal adaptations to opportunistic infections in woody plant degrading pathogens of *Botryosphaeriaceae*. *DNA Research*, 25, 87–102.

Yuan, M., Pok, B., Man, N., Ding, P. & Xin, X.F. (2021) PTI-ETI crosstalk: an integrative view of plant immunity. *Current Opinion in Plant Biology*, 62, 102030.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.