LtEPG1, a Secretory Endopolygalacturonase Protein, Regulates the Virulence of *Lasiodiplodia theobromae* in *Vitis vinifera* and Is Recognized as a Microbe-Associated Molecular Patterns

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ABSTRACT

The *Lasiodiplodia theobromae* genome encodes numerous glycoside hydrolases involved in organic matter degradation and conducive to pathogen infection, whereas their molecular mechanisms are still largely unknown. Here, we identified the glycoside hydrolase family 28 endopolygalacturonase LtEPG1 in *L. theobromae* and characterized its function in detail. LtEPG1 acts as a virulence factor during *L. theobromae* infection. Overexpression and silencing of *LtEPG1* in *L. theobromae* led to significantly increased and decreased lesion areas, respectively. Further, the high transcript level of *LtEPG1* during the infection process supported its virulence function. Polygalacturonase activity of LtEPG1 was substantiated by detecting its ability to degrade pectin. Furthermore, LtEPG1 functioned as microbe-associated molecular patterns during the infection process. Both transient expression of LtEPG1 in planta and infiltration of purified LtEPG1 triggered cell death in *Nicotiana*

benthamiana. Site-directed mutation of *LtEPG1* indicated that the enzymatic activity of LtEPG1 is independent from its elicitor activity. A protein kinase, KINβ1, was shown to interact in the yeast two-hybrid system with LtEPG1. This interaction was further confirmed in vitro using a pull-down assay. Our data indicate that LtEPG1 functions as a polygalacturonase and also serves as an elicitor with two independent mechanisms. Moreover, LtEPG1 may be able to manipulate host immune responses by regulating the KINβ1-mediated signal pathway and consequently promote its own successful infection and symptom development.

Keywords: cell death, elicitor activity, endopolygalacturonase activity, genetics and resistance, glycoside hydrolase family, *Lasiodiplodia theobromae*, mycology

The plant cell wall is the first layer encountered by fungal pathogens during their invasion into host plants. The basic structure of the plant cell wall is a complex of cellulose microfibrils embedded in a matrix consisting of hemicellulose, lignin, pectin, and structural proteins (Cosgrove 2005; Kubicek et al. 2014). Among these biopolymers, pectin, a heteropolysaccharide accounting for 35% of the primary cell wall, is a major constituent of the middle lamella of the primary cell wall of dicotyledonous plants (Kubicek et al. 2014; van der Vlugt-Bergmans et al. 2000; Voragen et al. 2009). Woody dicots, including many economically important plants, are significantly rich in pectin and its derivatives in their plant cell walls (Mellerowicz et al. 2001). Pectin not only is important for the integrity and rigidity of the plant cell wall and other plant tissues, but it also plays a vital role in the defense against wounding and plant pathogens (Mohnen 2008; Voragen et al. 2009).

Phytopathogenic fungi, especially those that do not possess specialized penetration structures, for example, *Botryosphaeria dothidea*, *Coniella vitis*, *Diplodia corticola*, *Lasiodiplodia theobromae*, and *Phyllosticta ampellicida* (Wilcox et al. 2015), produce diverse cell wall-degrading enzymes (CWDEs) to deconstruct the plant cell wall polymers, such as cellulose, pectin, and xylan. Furthermore, a considerable portion of CWDEs secreted by pathogens (Ma et al. 2015; Poinssot et al. 2003; Zhang et al. 2014) or even the decomposition products of cell walls have been identified as pathogen-associated molecular patterns during their interactions with host plants (Cheung et al. 2008; D'Ovidio et al. 2004; Hématy et al. 2009; Liu et al. 2015; Ma et al. 2015; Sakamoto et al. 2009; Yang et al. 2007). Based on sequence similarities, these CWDEs with pectin-degrading ability have been classified mainly into the glycoside hydrolase family 28 (GH28) (Henrissat 1991).

As an important member of CWDEs, endopolygalacturonases belong to the GH28 family and have been identified and characterized in various fungi (Herron et al. 2000; Kjølner and Struwe 2002; Reignault et al. 2007). For example, the importance of endopolygalacturonases functioning as virulence factors for fungal pathogenicity has been proven in *Alternaria citri* (Issshiki et al. 2001), *Aspergillus flavus* (Shieh et al. 1997), *Botrytis cinerea* (Kars et al. 2005; ten Have et al. 1998), *Claviceps purpurea* (Oeser et al. 2002), and *Fusarium oxysporum* (Durrands et al. 1988; Howell 1976; Mann 1962). In contrast, several studies have shown that endopolygalacturonases are not important for the infection process (Gao et al. 1996; Scott-Craig et al. 1990). These results indicate that endopolygalacturonases play multiple roles during the infection process. Although the functional mechanisms of endopolygalacturonases have been investigated in

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different organisms (Di Pietro and Roncero 1998; Isshiki et al. 2001; Kars et al. 2005; Poinssot et al. 2003; Shieh et al. 1997; ten Have et al. 1998), the molecular function of its ortholog in *L. theobromae* is still unclear.

L. theobromae, the causal agent of grape canker disease, has been described as a latent pathogen, and the lifestyle of *L. theobromae* can change from endophytic to pathogenic in the presence of external stimuli resulting from changing environmental conditions inside the host such as changes in host behavior or microbial equilibrium, or outside the hosts such as changes in climate or extreme environmental events (Chethana et al. 2016; Slippers and Wingfield 2007). Diseases caused by *L. theobromae* has been reported to bring a considerable yield loss to the grape industry, but the pathogenic mechanisms of this fungus are poorly characterized.

In our previous study, we identified 315 glycoside hydrolase family genes based on genome and transcriptome analyses in *L. theobromae* (Yan et al. 2018), among which 14 genes are GH28 family members, which is the highest number of GH28 family genes recorded for any fungi. In addition, 4 of 14 genes were up-regulated during infectious stages, suggesting that *L. theobromae* has an intense capacity for cell wall pectin degradation. Among these four genes, the transcript level of *LtEPG1* (*L. theobromae* endopolygalacturonase 1) was up-regulated significantly during the infection process. In this study, it was found that *LtEPG1* could be secreted into the extracellular space and function as a virulence factor. Furthermore, *LtEPG1* was confirmed to be able to degrade pectin from citrus peel, and it could also trigger the cell death of *Nicotiana benthamiana*. Additionally, site-directed mutation of *LtEPG1* showed that the elicitor activity of *LtEPG1* was independent of its enzymatic activity. Together, these results indicate that *LtEPG1* plays important roles in multiple aspects including pathogenesis, pectin degradation, and immune response regulation during the successful infection of *L. theobromae*.

MATERIALS AND METHODS

Fungal and bacterial isolates, culture conditions, and plant material. *L. theobromae* isolate CSS-01s was obtained from the Beijing Academy of Agricultural and Forestry Sciences, Beijing, China. Yeast strain YTK12 and *Burkholderia glumae* strain were obtained from Wenxian Sun at China Agricultural University. The *pET-32a* vector used in this study were obtained from Zejian Guo at China Agricultural University. Cultures were grown on potato dextrose agar (PDA [1 liter]: 200 g of potato, 20 g of dextrose, and 20 g of agar) medium at 28°C. All of the fungal cultures and bacterial cultures in the study were stored on PDA slants at 4°C and in 50% glycerol at -80°C, respectively. For RNA extraction, fungal mycelia were cultured in complete medium broth (CM: 0.6% [wt/vol] yeast extract, 0.3% [wt/vol] casein acid hydrolysate, 0.3% [wt/vol] casein enzymatic hydrolysate, and 1% [wt/vol] sucrose) in Erlenmeyer flasks on a shaking incubator at 160 rpm and 25°C for 2 days. For plasmid extraction and protein expression, bacteria were cultured in Luria-Bertani (LB: 1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 1% [wt/vol] NaCl at pH 7.5) broth supplemented with their respective antibiotics at 37°C in a shaking incubator at 160 rpm. Green shoots of *Vitis vinifera* 'Summer Black' were obtained from Yanqing, China, and the *N. benthamiana* plants were grown and maintained at 25°C in the controlled growth chamber at Beijing Academy of Agriculture and Forestry Sciences, Beijing, China. Agar and peptone were purchased from Bacto (Becton, Dickinson and Company, MD, U.S.A.), and yeast extracts were purchased from Oxoid (Oxoid Ltd., U.K.). All chemicals and reagents used in specific experiments were purchased from Sigma-Aldrich Co. (U.S.A.). Restriction enzymes, LA Taq polymerases, and T4 ligase were supplied by TaKaRa Bio, Inc. (Japan). *Escherichia coli* strains DH5 α and BL21 and *pMD18-T* simple blunt vectors (TaKaRa Bio Inc.) were used for DNA manipulations and amplification.

Bioinformatic analysis. The GH28 domain of *LtEPG1* was identified via pBlast against the Pfam database with the amino acid sequence of *LtEPG1* as the query sequence and the default settings of the server. The signal peptide of *LtEPG1* was predicted by the SignalP 4.1 server with the default settings (<http://www.cbs.dtu.dk/services/SignalP/>). Putative GH28 family protein sequences of botryosphaeriaceous species and their homologs from other closely related species were downloaded. Downloaded sequences were aligned using the default settings of MAFFT version 7 (Katoh and Toh 2008) (<https://mafft.cbrc.jp/alignment/server/>) and improved manually using BioEdit (Hall 1999). Maximum parsimony analyses were conducted for the GH28 family proteins in PAUP (phylogenetic analysis using parsimony) v.4.0b1037 (Swofford 2002) using the heuristic search option with tree bisection-reconnection branch swapping and 1,000 random sequence additions. Ambiguous regions in both alignments were excluded, and gaps were treated as missing data. Clade stabilities were assessed using a bootstrap analysis with 1,000 replications (Hillis and Bull 1993). Maxtrees were set to 1,000, branches of zero length collapsed, and all multiple parsimonious trees were saved. Phylogenetic trees were graphically viewed using Treeview v.1.6.6 (Page 1996).

Functional validation of the predicted signal peptide using yeast secretion assay. Functional validation of the predicted signal peptide of *LtEPG1* was performed within an invertase secretion-defective yeast (Gu et al. 2011). During the experiment, the encoding sequence of the *LtEPG1* signal peptide, a 0.2-kb fragment from the start codon of *LtEPG1*, was subcloned into the plasmid *pSuc2t7M13ori* (*pSUC2*), and the primers were listed in Table 1 (Jacobs et al. 1997). The resultant fusion plasmids were transformed into the invertase secretion-defective yeast strain YTK12 by the lithium acetate method described in Becker and Lundblad (1994). Transformants were plated on YPDA medium (1% yeast extract, 2% Difco peptone, 2% glucose, 0.003% adenine hemisulfate, and 2% agar), CMD-W medium (0.67% yeast nitrogen base without amino acids, 0.075% tryptophan dropout supplement, 0.1% glucose, 2% sucrose, and 2% agar) (Treco and Lundblad 1993), and YPRAA medium (1% yeast extract, 2% peptone, 2% raffinose, and antimycin A at 2 $\mu\text{g ml}^{-1}$) (Jacobs et al. 1997). The parental strain YTK12 and the strain transformed with *pSUC2* were used as negative controls. Plates were photographed after 4 days of incubation at 28°C.

Induction of cell death in *N. benthamiana*. The induction of cell death elicited by *B. glumae* on *N. benthamiana* leaves was performed (Sharma et al. 2013). The *LtEPG1* gene without the signal peptide encoding sequence and the stop codon was amplified using the primers listed in Table 1 and was subcloned into the vector *pEDV5* (Fabro et al. 2011). *B. glumae* competent cells were prepared by following the protocol given in Sharma et al. (2013). The confirmed constructs were transformed into the *B. glumae* competent cells by electroporation (Bio-Rad MicroPulser, Champaign, IL). Transformed strains were screened on LB agar supplemented with gentamycin at 25 mg liter $^{-1}$. *N. benthamiana* plants were infiltrated with bacterial inocula of positive transformants (suspended in a 0.9% NaCl) at optical density (OD) $_{600}$ = 0.4 using a 1-ml needleless syringe. The left half of the leaf was injected with *B. glumae* carrying the *pEDV* empty vector, and the right half was injected with *B. glumae* carrying *pEDV:LtEPG1*. The experiment was conducted independently three times, and each time, three leaves per plant and four plants were used as biological replicates. Plants were kept at 25°C in the controlled growth chamber at Beijing Academy of Agricultural and Forestry Sciences. Hypersensitive responses were observed within 3 days after infiltration.

Overexpression and RNAi vector construction of the *LtEPG1* gene and the pathogenicity tests. The full-length coding sequence (without the stop codon) of *LtEPG1* was amplified with primers given in Table 1 and subcloned downstream of the *Ptpc* promoter in a modified *pBluescript II KS* vector. The *LtEPG1*

gene fused with the modified *KS* vector was transformed into *L. theobromae* strain CSS-01s using the PEG/CaCl₂ transformation method (Yan et al. 2018). Transformants were screened using neomycin at 1,100 µg ml⁻¹ (Sigma-Aldrich, U.S.A.), and positive transformants were confirmed via quantitative real-time PCR (qRT-PCR). Then, the overexpression transformants of the *LtEPG1* gene were subjected to pathogenicity tests on detached *V. vinifera* 'Summer Black' green shoots with methods described previously (Yan et al. 2018). The inoculated grape shoots were maintained on an alternating dark/light cycle at 25°C in the controlled growth chamber for three days. The experiment was conducted independently three times, and each time, 10 shoots were used as biological replicates for each overexpressed transformant of *LtEPG1*. Three days postinoculation, lesion length was recorded for each transformant, and one-way analysis of variance (ANOVA) was used to analyze data obtained from the pathogenicity tests at a 5% significance level. The analysis was implemented in the MINITAB statistical program v.16.0 (Minitab Inc., U.S.A.). Shapiro-Wilk and Jarque-Bera normality tests were conducted for all datasets. The means of the different isolates were compared using Fisher's test at the 5% significance level.

For the RNAi vectors, the sense fragment and antisense fragment of *LtEPG1* gene were amplified using primers given in Table 1. Then, the amplified fragments were ligated and expressed under the *PtpC* promoter. The RNAi construct of *LtEPG1* gene was transformed into *L. theobromae* strain CSS-01s with the method used for the overexpression transformation. Subsequent pathogenicity tests and data analyses were performed using the same methods adopted for the overexpression experiment.

RNA extraction and quantitative real-time reverse transcription PCR analyses. Total RNA was extracted from vegetative hyphae that were cultured in liquid complete media at 28°C in a shaking incubator at 160 rpm for 2 days. Total RNA was isolated with Ambion Trizol Reagent (Life Technologies, U.S.A.) (Pham et al. 2011). The RNA concentration was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific, U.S.A.). Then, the isolated RNA (3 µg) was reverse transcribed to cDNA for the subsequent qRT-PCR experiment.

qRT-PCR was conducted for the overexpression and RNAi transformants of *LtEPG1* using gene specific primers listed in

Table 1 in a 7500 real-time PCR system (Applied Biosystems, Singapore) with SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio Inc., China). The *Actin* gene of *L. theobromae* was used as the internal reference. qRT-PCR was performed in 15-µl final volume consisting of 1.0 µl of cDNA, 0.5 µl of primer (10 µM), 0.3 µl of ROX reference dye, 5.2 µl of sterile water, and 7.5 µl of 2× SYBR Premix Ex Taq. The amplification conditions were as follows: 2 min for denaturation at 95°C, followed by 40 cycles of 95°C for 5 s, and 60°C for 35 s. Relative gene expression was calculated using the 2^{-ΔΔC_t} method. The experiment was conducted thrice with three independent replicates for each of the selected transformants.

Expression and purification of LtEPG1 and point mutation form. The coding sequence of LtEPG1 was obtained and ligated into the expression vector *pET-32a*. The point mutation forms of LtEPG1 were obtained by following the PCR-mediated splicing overlap extension approach as described in Horton (1995). Subsequently, the fusion construct was transformed into *E. coli* strain BL21 as explained by Sugantha Priya et al. (2010). A positive clone was cultured in 200 ml of LB broth at 37°C. When the OD₆₀₀ of the *E. coli* culture reached approximately 0.6, an appropriate amount of isopropyl-β-D-thiogalactoside (IPTG) was added at a final concentration of 0.2 mM to induce protein expression (Drew et al. 2001). Subsequently, the cultured bacteria were collected and sonicated on ice using the SONICS Vibra-cell Sonicator (Sonic & Materials, CT, U.S.A.). Cells were sonicated at 50% duty cycle 60 times, consisting of 4 s bursts with 2 s intervals. Then, the sonication mixture was centrifuged for 30 min at 12,000 rpm under 4°C. LtEPG1 existed in the supernatant and was further purified with Ni-NTA His bind resin (Qiagen, U.S.A.). A total of 5 ml of protein solution was obtained and 5 µl of protein was loaded into the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After running the gel, purified proteins were detected following Coomassie brilliant blue staining.

The purified proteins were adjusted to a final concentration of 700 nM and then agroinfiltrated onto 4-week-old *N. benthamiana* leaves. Plants were kept at 25°C in the controlled growth chamber, and cell death responses were observed within 5 days after infiltration. Then, *N. benthamiana* leaves were stained with lactophenol/ethanol/trypan blue (10 ml of lactic acid, 10 ml of

TABLE 1. Primers used in the current study

Primer name	Sequence	Remarks
Primers used in yeast complementary experiments		
LtEPG1FE	5'-ATGAATTCATGGTCAAGGTTTCCACCCT-3'	<i>EcoRI</i> site is underlined
LtEPG1RX	5'-TTCTCGAGGATGGCAGAGGCAGC-3'	<i>XhoI</i> site is underlined
pEDV vector construction		
LtEPG1FSal	5'-TAGTCGACGCCCCACTGAGCTC-3'	<i>SalI</i> site is underlined
LtEPG1RBam	5'-AAGGATCCGCAAGAGATGCCAGAGC-3'	<i>BamHI</i> site is underlined
Primers used for the overexpression vector construction		
LtEPG1FEco	5'-TCAAGCTTATGGTCAAGGTTTCCA-3'	<i>EcoRI</i> site is underlined
LtEPG1FHind	5'-TAGAATTCGCAAGAGATGCCAGAG-3'	<i>HindIII</i> site is underlined
Primers used for the RNAi vector construction: Sense strand		
RiLtEPG1SFPst	5'-AACTGCAGAAGGTTTCCACCCTCCTT-3'	<i>PstI</i> site is underlined
RiLtEPg1SRSal	5'-ATGTTCGACGACTTCAGGCCAGTGAGA-3'	<i>SalI</i> site is underlined
Primers used for the RNAi vector construction: Antisense strand		
RiSLtEPG1ASEF	5'-AAGAATCCAAGGTTTCCACCCTCCTT-3'	<i>EcoRI</i> site is underlined
RiSLtEPG1ASXhF	5'-ATCTCGAGGACTTCAGGCCAGTGAGA-3'	<i>XhoI</i> site is underlined
Primers used for the transcript level analyses of <i>LtEPG1</i> gene in overexpression and RNAi transformants of <i>LtEPG1</i>		
QLtEPG1F	5'-TTCGGTTACAAGGAGTGGGAG-3'	–
QLtEPG1R	5'-GCTGTGGGCGTAGAAGAACT-3'	–
Primers used for the construction of protein expression vector		
LtEPG1BamHIF	5'-TTGGATCCATGGCCCCACTGAGCT-3'	<i>BamHI</i> site is underlined
LtEPG1SmaIR	5'-AACCCGGGGCAAGAGATGCCAGA-3'	<i>SmaI</i> site is underlined
Primers used for the construction of LtEPG1-pGBKT7 fusion construction		
LtEPG1BDF	5'-CGGAATTCATGGTCAAGGTTTCCACCC-3'	<i>EcoRI</i> site is underlined
LtEPG1BDR	5'-CGGGATCCTTAGCAAGAGATGCCAGAG-3'	<i>BamHI</i> site is underlined
Primers used for the confirmation of positive transformants screened by yeast two-hybrid		
Y2HPF	5'-GCACCTATTCGATGATGAAGATACC-3'	–
Y2HPR	5'-GCACGATGCACAGTTGAAGTG-3'	–

glycerol, 10 g of phenol, 30 ml of absolute ethanol, and 10 mg of trypan blue dissolved in 10 ml of distilled water) via boiling in lactophenol/ethanol/trypan blue solution for 5 min, followed by destaining in chloral hydrate at 2.5 g ml⁻¹.

Polygalacturonase activity assay. According to Miller (1959), polygalacturonase activity was determined by measuring the product absorbency at 575 nm caused by the release of reducing sugars from citrus pectin. Three separate reactions were conducted for a series of different enzyme volumes (0.2, 0.4, 1, 5, and 10 µl), with each volume represented by three replicates. The concentration of the enzyme was measured by flowing the Bradford assay (Bio-Rad Protein Assay) according to the manufacturer's instructions. The reaction mixtures consisted of (i) 250 µl of citrus pectin at 2 mg ml⁻¹ (Sigma-Aldrich, China) dissolved in 0.1 M sodium acetate buffer (pH 5.0) and the enzyme (0.1 mg ml⁻¹), (ii) 250 µl of glucose at 2 mg ml⁻¹ and the enzyme (0.1 mg ml⁻¹), and (iii) 125 µl of citrus pectin at 2 mg ml⁻¹ dissolved in 0.1 M sodium acetate buffer (pH 5.0), 125 µl of glucose at 2 mg ml⁻¹, and the enzyme (0.1 mg ml⁻¹). The mixture was incubated at 45°C for 30 min, and the reaction was stopped with the addition of 750 µl of di-nitro salicylic acid reagent, boiled for 5 min, and cooled on ice. Then, the absorbance of each mixture was measured in an Ultrospec 2100 pro UV/Visible Spectrophotometer (Amersham Biosciences, Sweden) at 575 nm.

Fig. 1. Functional validation of the predicted signal peptide of the LtEPG1 (*Lasiodiplodia theobromae* endopolygalacturonase 1) protein. **A**, Schematic diagram of LtEPG1. **B**, Functional detection of the signal peptide of the LtEPG1. The encoding sequence of the predicted signal peptide of LtEPG1 was fused upstream of the truncated form of the yeast invertase gene that lacked its own native signal peptide sequence to engineer the fusion construct *pSUC2:LtEPG1*. Yeast strain YTK12 transformed with the *pSUC2:LtEPG1* fusion vector was cultured on YPDA (1% yeast extract, 2% Difco peptone, 2% glucose, 0.003% adenine hemisulfate, and 2% agar), CMD-W (0.67% yeast nitrogen base without amino acids, 0.075% tryptophan dropout supplement, 0.1% glucose, 2% sucrose, and 2% agar), and YPRAA (1% yeast extract, 2% peptone, 2% raffinose, and antimycin A at 2 µg ml⁻¹) media and photographed after 4 days of incubation. All yeast strains grew on YPDA. Transformants carrying the *pSUC2* fusion plasmid grew on CMD-W. Only transformants whose invertase could be secreted into the extracellular space grew on YPRAA plates. YTK12 denotes the parental strain. *pSUC2:Avr1b*-YTK12 was used as the positive control, and *pSUC2:Mg87*-YTK12 was used as a negative control. *pSUC2:LtEPG1*-YTK12 denotes *LtEPG1* transformants.

Yeast two-hybrid assay. The ORF of the *LtEPG1* gene was cloned into the *pGBKT7* vector using the primers shown in Table 1 to construct the bait vector *pGBKT7-LtEPG1*. The bait plasmid was transformed into yeast strain AH109 by the lithium acetate method as described in Becker and Lundblad (1994). The grape cDNA library screening was performed with *pGBKT7-LtEPG1* as the bait by following the Matchmaker Gold Yeast Two-Hybrid System (Clontech, TaKaRa, China). The fished transformants were selected on synthetic dropout (SD/-Leu/-Trp/-His) medium, and the interaction between the bait protein and prey target was further confirmed by yeast two-hybridization. Primers are listed in Table 1.

MBP pull-down assays. The LtEPG1 and KINβ1 were cloned into *pGEX-4T-1* and *pMAL-C4X* vectors and expressed in *E.coli* BL21 to produce recombinant proteins. Protein purification and MBP pull-down assays were performed with the method described in a previous study (Liu et al. 2011).

RESULTS

LtEPG1 belongs to the GH28 family and is widely distributed across pathogenic fungal taxa. Based on genome and transcriptome analyses, it was found that four genes belonging to the GH28 family were up-regulated during the infection process. One of them, with a significantly elevated transcription level, was identified and referred to as *LtEPG1* (Yan et al. 2018). The 20 amino acids located at the N-terminal of LtEPG1 were predicted to be the signal peptide of LtEPG1 with SignalP 4.1 Server (Fig. 1A) (Petersen et al. 2011). Searching against the Pfam database with LtEPG1 as the query showed that LtEPG1 contains a Glyco_hydro_28 domain (PF00295) (Fig. 1A). Furthermore, conserved regions among the LtEPG1 protein and its closely related endopolygalacturonases were identified by amino acid sequence alignment (Supplementary Fig. S1). In the phylogenetic analysis, LtEPG1 was found to be clustered together with the other GH28 family proteins (Yan et al. 2018). The phylogenetic relationship between LtEPG1 and its homologs confirmed that LtEPG1 belongs to the same clade as another two putative endopolygalacturonases from botryosphaeriaceous pathogens *Diplodia seriata* and *D. corticola* (Supplementary Fig. S2).

After *LtEPG1* was cloned, we used the amino acid sequence of LtEPG1 as a query in a blastp search against the *L. theobromae* proteome for other orthologs. Interestingly, three more proteins with the accession numbers KAB2578544.1, KAB257894.1, and KAF4546527.1 were identified to share 82.38, 77.78, and 77.51% amino acid identities with LtEPG1, with 94, 100, and 100% coverages, respectively, and all of these proteins predicted to be polygalacturonases (Supplementary Fig. S3).

LtEPG1 encodes a secretory endopolygalacturonase protein. To validate the predicted exocrine ability of LtEPG1, an effective and efficient method of a trapping signal sequence was adopted, which has been widely used to identify the signal peptide of secretory proteins (Gu et al. 2011; Jacobs et al. 1997). To identify the signal sequence of *LtEPG1*, we engineered an *LtEPGISP-invertase* fusion construct, in which the signal peptide of LtEPG1 was introduced one amino acid before the truncated moiety of yeast invertase whose signal peptide had been cleaved. A constructed fusion vector (*pSUC2:LtEPG1*) with positive (*pSUC2:Avr1b*) and negative controls (*pSUC2:Mg87*) was transformed into the *SUC2*-yeast strain YTK12. As shown in Figure 1B, all of the yeast strains grew on YPDA medium, and yeast transformants carrying the *pSUC2* vector grew on CMD-W medium. However, only transformants *pSUC2:LtEPG1*-YTK12 carrying the LtEPG1 signal peptide and positive control containing *pSUC2:Avr1b* were able to rescue the growth of parental strain YTK12 on YPRAA plates, indicating that LtEPG1 has a functional signal peptide that can lead to the secretion of yeast invertase.

LtEPG1 is required for the full virulence of *L. theobromae* in *V. vinifera*. Based on our previous transcriptome analysis (Yan et al. 2018), the transcript level of *LtEPG1*

during the infectious stage was significantly higher ($\sim 4\times$) than that of vegetative mycelia. To determine the predicted contribution of *LtEPG1* to *L. theobromae* virulence, expression patterns of *LtEPG1* were analyzed during different infection stages, including the mycelia cultured in liquid CM medium, and 12, 24, 48, and 72 h postinoculation on wounded grape shoots. Results combined with the statistical analysis showed that the expression of *LtEPG1* was significantly increased during early infection stages (Fig. 2).

To functionally characterize *LtEPG1*, the *LtEPG1* gene was overexpressed and silenced via PEG-mediated transformation in *L. theobromae* (Fig. 3A and B). The positive transformants were screened on neomycin-containing plates and confirmed by qRT-PCR analysis (Fig. 3E). Phenotypic analyses show that *LtEPG1* had no significant effect on colony morphology and vegetative growth on PDA (Fig. 3C and D).

Further virulence analysis was also performed by inoculating equal-diameter mycelial plugs from wild-type, overexpression transformants, and RNAi transformants onto green shoots of *V. vinifera* ‘Summer Black’, which is susceptible to *L. theobromae*. The overexpression transformants of *LtEPG1* showed evidently elevated pathogenicity relative to that in the wild type, and the RNAi transformant of *LtEPG1* exhibited significantly decreased pathogenicity in comparison with that in the wild type. After 72 h postinoculation (hpi), the overexpression transformants displayed longer lesions with an average length of 5.12 cm, and the RNAi transformants, by contrast, showed smaller lesions with an average length of 1.53 cm, compared with the wild type, which has the average length of 3.56 cm (Fig. 3F and G). Taken together, these results indicate that *LtEPG1* is important for the virulence and symptom development of *L. theobromae*.

LtEPG1 is an endopolygalacturonase. *LtEPG1* has been predicted to be an endopolygalacturonase based on blastp analysis against the NCBI database, so we decided to investigate its enzymatic activity. Therefore, recombinant *LtEPG1* was isolated from *E. coli* BL21 that carried the *pET-32a:LtEPG1* fusion construct and was further purified with Ni-NTA beads. Purified proteins were detected by SDS-PAGE and visualized with Coomassie staining (Fig. 4A). Next, purified *LtEPG1* protein was assayed for endopolygalacturonase activity using glucose and citrus pectin as substrates. According to the assay, the absorbance of reaction product derived from purified *LtEPG1* and citrus pectin gradually increased at 575 nm when the volumes of purified *LtEPG1* added to the citrus pectin were gradually increased (Fig. 4B). However, no obvious difference in absorbance was detected with increasing enzyme volumes when glucose was used as the substrate. Due to the strong reaction between glucose and di-nitro salicylic acid, a higher absorbance was detected at 575 nm compared with that of citrus pectin. Similarly, in the presence of both substrates, no significant changes were observed in absorbance at 575 nm. The latter two results were expected since glucose has been shown to be able to repress polygalacturonase activity (Li et al. 2004). Since the absorbance values of the latter two reactions were beyond the measurable range, products of the latter two reactions were diluted before measuring the absorbance. These results indicate that *LtEPG1* functions as an endopolygalacturonase with citrus pectin as the substrate.

LtEPG1 from *L. theobromae* can induce cell death in *N. benthamiana*. To assess whether *LtEPG1* could elicit cell death response, transient expression of *LtEPG1* in *N. benthamiana* was tested. We engineered the fusion vector *pEDV:LtEPG1* and transformed it into *B. glumae* competent cells, which has been reported to be able to induce cell death when transiently expressed in *N. benthamiana* (Sharma et al. 2013). Transformed bacteria carrying *pEDV:LtEPG1*-Bg, *pEDV*-Bg, and empty *pEDV* were agroinfiltrated into *N. benthamiana* leaves, respectively. Results showed that *pEDV:LtEPG1*-Bg triggered a stronger cell death response 3 days postinfiltration compared with *pEDV*-Bg, and empty *pEDV* barely induce cell death (Fig. 5A).

To further confirm *LtEPG1* could trigger a hypersensitive response in *N. benthamiana*, recombinant *LtEPG1* was purified and then infiltrated into *N. benthamiana* leaves for cell death detection. Buffer was infiltrated as a control. As indicated in Figure 5C, *LtEPG1*-induced cell death in *N. benthamiana* leaves could be observed 5 days post protein infiltration. The results indicated that the prokaryotically expressed *LtEPG1* could trigger significantly visible cell death similar to the transient expression of *LtEPG1* in planta.

Enzymatic activity of *LtEPG1* is not required for its elicitor activity. As purified *LtEPG1* has been confirmed to be an endopolygalacturonase and can also elicit the cell death response in *N. benthamiana*, further investigation was carried out to test whether the enzymatic activity of *LtEPG1* is necessary for its elicitor activity. Amino acid sequence alignment of available polygalacturonases from plants, fungi, and bacteria showed that a total of eight amino acid residues of *LtEPG1* are strictly conserved among these polygalacturonase orthologs. These eight residues are Asn¹⁸², Asp¹⁸⁴, Asp²⁰⁵, Asp²⁰⁶, His²²⁷, Gly²²⁸, Arg²⁶⁰, and Lys²⁶². To assess their importance for enzymatic activity and elicitor activity, six out of eight conserved residues—Asp¹⁸⁴, Asp²⁰⁵, Asp²⁰⁶, His²²⁷, Arg²⁶⁰, and Lys²⁶²—were mutagenized to Glu, Glu, Glu, Ala, Asn, and Asn, respectively (Supplementary Fig. S4). The mutated forms (D184E, D205E, D206E, H227A, R260N, and K262N) were expressed in *E. coli* and detected in SDS-PAGE (Fig. 5B). No differences were observed in the molecular weight between *LtEPG1* and its mutated *LtEPG1* forms (Fig. 5B). Polygalacturonase assays showed that the reaction products of pectin with mutated forms of *LtEPG1* were hardly detected by di-nitro salicylic acid reagent, suggesting the enzymatic activity of mutated proteins was almost abolished or insignificant. These results indicate that these conserved residues are necessary for the enzymatic activity of *LtEPG1*.

To examine whether these conserved residues also affect the elicitor activity of *LtEPG1*, purified mutation forms of the *LtEPG1* protein were infiltrated into the leaves of *N. benthamiana*. Total mutated proteins induced visible cell death in *N. benthamiana* (Fig. 5C) and the cell death lesion was detectable by trypan blue staining (Fig. 5D). Taken together, these results indicate that the elicitor activity of *LtEPG1* is independent of its enzymatic activity.

Fig. 2. Transcript levels of *LtEPG1* at mycelial and different infection stages. Transcript levels of glycoside hydrolase family 28 endopolygalacturonase gene were assessed at the mycelial stage (MY), 12, 24, 48, and 72 h postinoculation in *Vitis vinifera* shoots using quantitative real-time PCR. Expression data were normalized by *actin* gene and calibrated against transcript level of the mycelial stage. Means and standard deviations from three biological replicates are shown. Asterisks (**) indicate significant differences as measured by least significant difference test ($P < 0.01$).

Fig. 3. *LiEPG1* contributes to the virulence of *Lasiodiplodia theobromae* in *Vitis vinifera* shoots. **A**, Schematic diagram of the *LiEPG1* gene overexpression vector. **B**, Schematic diagram of the *LiEPG1* gene silencing vector. **C**, Radial growth rates of wild-type CSS-01s, overexpression transformants, and silencing transformants of *LiEPG1* after 24 and 48 h at 28°C. Bars indicate the standard deviations of three biological replicates. One-way analysis of variance (ANOVA) was performed for statistical analysis. Isolates that do not share same letter exhibit significantly different growth rates among the isolates. **D**, Phenotypes of wild-type CSS-01s, overexpression transformants, and silencing transformants of *LiEPG1* after 48 h at 28°C. **E**, The transcript level of *LiEPG1* in wild-type CSS-01s, overexpression transformants, and silencing transformants. *Actin* was used as the endogenous control. Means and standard deviations of three biological replicates are shown. One-way ANOVA was performed for statistical analysis. Isolates that do not share same letter exhibit significantly different gene expression among the isolates. **F**, Lesion lengths of the wild-type CSS-01s, overexpression transformants, and silencing transformants of *LiEPG1*. Bars indicate the standard deviations of 10 biological replicates. One-way ANOVA was performed for statistical analysis. Isolates that do not share same letter exhibit significantly different lesion lengths. **G**, Pathogenicity assay of wild-type, overexpression transformants, and silencing transformants of *LiEPG1* in *V. vinifera* green shoots. OE-*LiEPG1*-1 and OE-*LiEPG1*-2 denote the *LiEPG1* overexpression transformants, and RNAi-*LiEPG1*-1 and RNAi-*LiEPG1*-2 denote the *LiEPG1* silencing transformants.

LtEPG1 interacts with KIN β 1. As LtEPG1 was confirmed to be a secretory protein, we conducted a yeast two-hybrid screen against the grape cDNA library with LtEPG1 as the bait to fish the potentially interacting targets of LtEPG1. As shown in Supplementary Figure S5, LtEPG1 did not display the transcriptional activation activity when the bait vector was expressed in yeast. Further, 16 proteins potentially interacting with LtEPG1 were identified as shown in supporting information in Supplementary Table S1. Among the 16 potential candidates, one protein named KIN β 1, was selected for further analysis (Fig. 6A).

To confirm the interaction of LtEPG1 and KIN β 1, we expressed their full-length cDNA sequences in yeast. Results indicated that LtEPG1 indeed interacted with KIN β 1. We further confirmed interaction between LtEPG1 and KIN β 1 with MBP pull-down assays. Pull-down assays using the recombinant GST-LtEPG1 and MBP-KIN β 1 showed that two proteins interacted with each other in vitro (Fig. 6B).

The homolog of KIN β 1 was reported to be a regulatory subunit of the SNF1-related protein kinase complex (Tabata et al. 2000), but the functional analyses related to immune response in grape is barely reported. Here, the transcript level of *KIN β 1* at different infection points was determined by qRT-PCR analysis (Supplementary Fig. S6). Surprisingly, the transcription level of *KIN β 1* was significantly decreased during the early infectious stages, suggesting that KIN β 1 may function as an immune response regulatory factor, but its functional mechanisms need to be investigated in depth.

In summary, we assume a working model of LtEPG1 during the infection processes. LtEPG1 disrupts the plant cell wall components to facilitate the infection of *L. theobromae*; LtEPG1 interacts with KIN β 1, but the regulatory mechanisms of the interaction on the downstream signal pathways including burst of reactive oxygen species, increased expression of defense-related genes, and immune response need to be further investigated.

DISCUSSION

During plant–pathogen interactions, the plant cell wall represents a major barrier for the successful invasion of pathogens, and conversely, pathogens possess a diversified arsenal known as CWDEs to overcome these obstacles. Until now, a plethora of CWDEs have been reported to be conducive to the depolymerization of plant cell wall components, including cellulose, hemicellulose, lignin, pectin, xylan, and xyloglucan (Kubicek et al. 2014). Advances in genomic, transcriptomic and proteomic research have revealed large amounts of CWDE families that are

specifically present or magnified in different pathogenic fungi. Previous studies also showed that CWDEs were significantly essential, not only for the pathogens that lack specialized infection structures but also for many phytopathogenic fungi that depend on mono- and oligosaccharides liberated from plant tissues for their growth and reproduction (Gibson et al. 2011; Kubicek et al. 2014). Recent research (Yan et al. 2018) confirmed that *L. theobromae* possess a large number of CWDE-encoding genes, but the functional mechanisms of these CWDEs are still unknown.

Fig. 4. The endopolygalacturonase activity analysis of LtEPG1. **A**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to confirm the expression of LtEPG1 in *Escherichia coli*. The gel was stained with Coomassie brilliant blue and scanned. Asterisk denotes the target protein. **B**, Results of endopolygalacturonase activity. Enzyme activity is reported as the increase in absorbance at $A_{575\text{ nm}}$ from the release of reducing sugars from pectin. Bars indicate the standard deviation of three replicates.

Fig. 5. LtEPG1 expression induces defense responses in *Nicotiana benthamiana* leaves. **A**, Transient expression of *LtEPG1* elicits increased cell death responses in *N. benthamiana* leaves after infiltration with *Burkholderia glumae* harboring *pEDV*-Bg and *pEDV*:*LtEPG1*-Bg. Opposite areas of the leaf surface were infiltrated with *pEDV*-Bg and *pEDV*:*LtEPG1*-Bg. Representative cell death symptoms were photographed 3 days postinoculation. **B**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis to confirm the expression of the protein LtEPG1 and its mutated forms in *Escherichia coli*. No differences in molecular weight were observed between LtEPG1 and its mutated LtEPG1 forms. WT denotes the LtEPG1 protein and D184E, D205E, D206E, H227A, R260N, and K262N denote the mutated forms of the LtEPG1 protein. **C**, Cell death in *N. benthamiana* leaves was assessed by visual inspection 5 days postinfiltration with purified LtEPG1 proteins and their mutated forms. LtEPG1^{D184E}, LtEPG1^{D205E}, LtEPG1^{D206E}, LtEPG1^{H227A}, LtEPG1^{R260N}, and LtEPG1^{K262N} denote the mutated forms of the LtEPG1 protein. All of the mutated forms induced visible cell death in *N. benthamiana* leaves. **D**, Cell viability measured by trypan blue staining.

Here, we identified a CWDE LtEPG1 in *L. theobromae*, which belongs to GH28 based on amino acid sequence alignment. Yeast complementary experiments showed the signal peptide of LtEPG1 was able to guide the secretion of yeast invertase, which indicated that LtEPG1 was secreted into extracellular spaces during the pathogen–plant interaction process. Amino acid sequence analyses showed that LtEPG1 has a GH28 domain, suggesting that LtEPG1 can function as an endopolygalacturonase. Enzymatic activity evaluation confirmed that LtEPG1 has enzymatic activities with pectin as its substrate, and the hydrolytic reaction can be repressed by glucose, which is consistent with previously reported results (Di Pietro and Roncero 1998; Kubicek et al. 2014). The hydrolysis ability of LtEPG1 suggests that *L. theobromae* may be able to decompose the cell wall component of host plants during the infection process to support disease development.

Moreover, many polygalacturonases have been reported as virulence factors in plant pathogens, such as Pgl in the wilt pathogen *F. oxysporum* (Di Pietro and Roncero 1998), Acp1 in the citrus pathogen *Alternaria citri* (Isshiki et al. 2001), and Bcpg1 to Bcpg6 in the grapevine pathogen *B. cinerea* (Kars et al. 2005; Poinssot et al. 2003; ten Have et al. 1998). Similar to previous studies, LtEPG1 was also confirmed to be a virulence factor by overexpression and RNAi in combination with pathogenicity tests. LtEPG1, however, had no influence on the phenotypes, including fungal morphology and colony characteristics, suggesting that LtEPG1 may mainly function during the infection process. Furthermore, the involvement of LtEPG1 in the infection process was further confirmed by qRT-PCR. The increased transcript level of *LtEPG1* at 12 hpi indicates that LtEPG1 has more active roles during the early infection stages, most probably activating its putative polygalacturonase activity with plant cell wall components as its substrate. Symptoms became visible at 24 hpi on the green shoots. This timing lacks behind *LtEPG1* gene expression probably because several mechanisms need to work together after gene expression for physical damage to appear. Moreover, eight conserved amino acids were also identified in LtEPG1 according to amino acid sequence alignment against its orthologs. Surprisingly, single site-substitution of these amino acid residues led to almost complete loss of enzymatic activity, which concurs with the documented results in other fungi (van Santen et al. 1999).

Many glycoside hydrolase family proteins, including XEG1 of *Phytophthora sojae* (Ma et al. 2015), chitinases and xylanases of many fungal taxa (Ron and Avni 2004; Xu et al. 2016), INF1 of *P. capsica* and *P. infestans* (Kamoun et al. 1998; Liu et al. 2015), Avr2, Avr4, Avr4E, Avr5, and Avr9 of *Cladosporium fulvum* (de Wit 2016), and fungal endopolygalacturonases (Zhang et al. 2014), have been confirmed to be able to trigger plant defensive responses (Misas-Villamil and van der Hoorn 2008). In this study, LtEPG1 was also confirmed to induce the cell death, which suggest that LtEPG1 may function as a microbe-associated molecular pattern (MAMP) during the invasion process of *L. theobromae*.

Furthermore, one protein, KINβ1, was confirmed to interact with LtEPG1 by yeast two-hybridization. KINβ1 was reported to be a regulatory subunit of the probable trimeric SNF1-related protein kinase (SnRK) complex, which has been previously identified to have a key role in the signal transduction cascade regulating gene expression and carbohydrate metabolism in higher plants (Emanuelle et al. 2015). Gene expression analysis showed that *KINβ1* was considerably down-regulated during infectious stages, which was completely the opposite of the expression pattern of LtEPG1, suggesting that *KINβ1* may be negatively regulated during the infection. Based on the results mentioned above, we inferred that LtEPG1 may be able to manipulate the expression of resistance-related genes and the host immune response by interfering with the KINβ1-mediated signaling pathway. Substitution of eight conserved residues resulted in almost entire loss of the polygalacturonase activity, whereas their ability to trigger cell death in *N. benthamiana* was not affected, indicating the function of LtEPG1 as a MAMP was independent of its enzymatic activity. The enzymatic activity of LtEPG1 is related to these key amino acids, and this explains the loss of activity in the mutated forms of the enzyme. As suggested by the above results, the elicitor activity of LtEPG1 may relate to its interaction with KINβ1. Since the point mutated forms of LtEPG1 still function as elicitors, they may still be able to interact with KINβ1.

LtEPG1 was found to be a virulence factor in *L. theobromae*. Furthermore, the polygalacturonase activity of LtEPG1 was independent of its elicitor activity. Interestingly, it was found that LtEPG1 interacted with KINβ1 in *V. vinifera*. Given the important roles LtEPG1 played during the infection process, further studies are warranted on LtEPG1 and KINβ1, specifically on the regulatory pathways.

Fig. 6. LtEPG1 interacts with the *Vitis vinifera* KINβ1 protein. **A**, LtEPG1 interacts with KINβ1 confirmed by yeast two-hybrid assay. Yeast cells expressing the prey construct of *KINβ1* and bait construct of *LtEPG1* were assayed for growth on SD/-Leu/-Trp/-His plates and for β-galactosidase activities. Yeast transformants expressing the empty prey construct *pGADT7* with empty bait construct *pGBKT7* were used as the negative control. Yeast cells expressing the *pGADT7-T* prey construct with the *pGBKT7-53* bait construct were used as the positive control. Plates were photographed 3 days after inoculation. **B**, Protein GST-LtEPG1 interacts with MBP-KINβ1 using MBP pull-down assays. The recombinant proteins GST-LtEPG1 and MBP-KINβ1 expressed in *E. coli* were subjected to MBP pull-down analyses. The interacting proteins were detected via immunoblotting analyses.

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