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Full Length Article

Overexpression of Cotton Laccase Gene *LAC1* Enhances Resistance to *Botrytis cinereal* in Tomato Plants

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Abstract

Laccase is a glycoprotein oxidase containing four copper ions found in bacteria, fungi, insects and plants. In higher plants, laccase is mainly involved in the synthesis of lignin and the regulation of the content of phenolic substances in plants to enhance the plant's defense against diseases and pests. In this study, we constructed an overexpression vector harboring the *Gossypium hirsutum LAC1* gene and introduced it into tomato (*Solanum lycopersicum* L.). The laccase activity, gray mold resistance and lignin level in transgenic tomato overexpressing *GhLAC1* were analyzed using the wild-type as the control. The former had significantly higher laccase activity and gray-mold resistance levels compared with those of wild-type. Additionally, compared with the control plants, the lignin contents in the leaves and stems of the transgenic tomato were significantly higher. Scanning electron microscopy was used to observe the cross sections of wild-type and transgenic tomato stem segments and the cell wall near the xylem catheter of the latter was significantly thicker than that of the former and the outline was more distinct. Thus, the *GhLAC1* gene significantly increased the lignin content in tomato, thickened the cell walls and strengthened physical defenses, thereby increasing tomato resistance to gray mold.

Keywords: *GhLAC1*; Laccase; Transgenic tomato; Lignin; Gray mold

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown vegetables in the world and gray mold, which is caused by *Botrytis cinerea* Pers., is a serious disease resulting in large reductions in tomato production (Ma *et al*. 2018). Currently in China, gray mold is commonly controlled by spraying chemical fungicides, which has led to resistant strains of the pathogenic fungi and a certain degree of pesticide pollution owing to unreasonable application practices (Zhao *et al*. 2014; Liu *et al*. 2017a). The breeding of resistant varieties is an effective method for plant disease prevention and control, but there are limited reports on the breeding of tomato resistant to *B. cinerea* Pers. Transgenic breeding is a commonly used effective method. In Powell *et al*. (2000) study of the polygalacturonase inhibitor protein gene (*PGIP*) of pears (*Pyrus* spp.), transgenic *pPGIP*-expressing tomato was more resistant to gray mold than the control. Coego (2005) transformed an *Arabidopsis thaliana* transcription factor gene into tomato, resulting in a new tomato strain resistant to *B. cinerea* Pers. Study shown that the tomato calcineurin B-like gene *CBL1* can regulate the resistance of tomato to gray mold by affecting resistance-related transcription factors (Wang *et al*. 2016). Our research group transformed the *Eucommia ulmoides* Oliver chitinase gene *CHIT1* into tomato and found that the transgenic tomato had greater disease resistance than the untransformed control (Guo *et al*. 2016).

Laccase is a glycoprotein that has a wide range of substrates and a high catalytic activity (Wang *et al*. 2017). It can be divided into two groups, plant and fungal laccases, based on its source (Deng *et al*. 2017). Laccase has been reported in many plants, including *A. thaliana*, cotton (*Gossypium* spp.), tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), maize (*Zea mays*) and *Populus trichocarpa* (Kiefer-Meyer *et al*. 1996; Liang *et al*. 2006; Wang *et al*. 2008; Berthet *et al*. 2011; Cesarino *et al*. 2013; Cao 2016). Research on plant laccases has mainly focused on the polymerization of lignin (Liu *et al*. 2017b). In *Arabidopsis*, the *AtlLAC4* and *AtlLAC17* genes were found to contribute to constitutive lignification by studying the double mutants *lac4-1 lac17* and *lac4-2 lac17* (Berthet *et al*. 2011). Ranocha *et al*. (2002) researched three independent populations of antisense transgenic poplar plants and determined that the total soluble phenol content in the *lac3*AS line increased by two to three times. Moreover, they observed that the inhibition of *lac3* led to dramatic alterations in xylem fiber cell walls. Laccases are also

involved in lignin synthesis in the grass crop, *Brachypodium distachyon*, and the lignin content of a *BdLAC5*-misregulated *Bd4442* mutant line was reduced (Wang *et al*. 2015). In addition, the laccase gene has been used to alter plant resistance to fungi, bacteria and insects. Transgenic tomatoes harboring the potato laccase gene show a significant increase in bacterial speck disease resistance compared with control tomatoes (Li and Steffens 2002). Wu discovered that the cotton (*Gossypium hirsutum*) laccase gene, *GhLAC1*, is involved in the lignin synthesis of cotton xylem and can accelerate the xylem lignification process, which is one reasons why this gene can enhance cotton's resistance to diseases and insect pests (Wu 2014). Recently, *GhLAC1* was shown to increase cotton resistance to *Verticillium dahliae* and cotton bollworm by strengthening lignification and mediating jasmonic acid (JA) biosynthesis (Hu *et al*. 2018). Zhang *et al*. (2019) determined that the cotton laccase gene *LAC15* enhances Verticillium wilt resistance by increasing defense-induced lignification, as well as levels of arabinose, xylose and lignin components in the cell walls of plants. Thus, laccases participate in the synthesis of lignin in plants and strengthen plant defense systems.

In this study, the cotton laccase gene *LAC1* was genetically transformed into tomato, and a new tomato germplasm resistant to gray mold was obtained. *GhLAC1* enhanced tomato resistance to gray mold by increasing the cell wall's lignin content, resulting in its improved strength. The results increase our knowledge of how plants strengthen disease defenses through lignification and provides theoretical support for future research on the detailed mechanisms of plant disease resistance.

Materials and Methods

Experimental material

Tomato (*S. lycopersicum*) 'Micro-Tom' was provided by our laboratory (Guizhou Key Laboratory of Agricultural Bioengineering). The seeds were sterilized and sown in germination medium [Murashige and Skoog (MS) + 20 g/L sucrose + 9 g/L agar powder], placed in a thermostatic plant tissue culture room, and cultured at 28°C with a 16-h light/8-h dark photocycle (Pan *et al*. 2010).

Construction of the plant overexpression vector

The sequence of the cotton laccase gene *LAC1* (GenBank: KT290561.1) was retrieved from NCBI and pGM626 (Guizhou Key Laboratory of Agricultural Bioengineering, Guizhou University) was used as the initial vector to construct the *Act1* promoter-driven *GhLAC1*-containing plant expression vector PGM626-Act1-*GhLAC1*. The construction of PGM626-Act1-*GhLAC1* was completed by Shanghai Xuguan Company.

Tomato transformation and identification of transgenic plants

The plasmid pGM626-Act1-*GhLAC1* was transformed into *Agrobacterium* using the freeze-thaw method and positive resulting colonies were identified by colony PCR. PCR amplification was carried out using the designed verification primers (Table 1), and the target amplicon size was 399 bp.

The *Agrobacterium tumefaciens*-mediated transformation method used was that of Guo *et al*. (2016). After the sterilized Micro-Tom seeds germinated on MS medium, the tomato cotyledons were cut and placed on the preculture medium (MS + 20.0 g/L sucrose + 9.0 g/L agar powder + 2.0 mg/L 6-BA + 0.5 mg/L IBA + 100 μmol/L AS), and cultured in the dark at 28°C for 2 d. Then, the cotyledon explants were subjected to *Agrobacterium* infection solution for 8 min and transferred to the co-cultivation medium (MS + 20.0 g/L sucrose + 9.0 g/L agar powder + 2.0 mg/L 6-BA + 0.5 mg/L IBA + 100 μmol/L AS) and cultured in the dark at 28°C dark for 3 d. After transformation, the cotyledon explants were placed on screening medium (MS + 20.0 g/L sucrose + 9.0 g/L agar powder + 2.0 mg/L 6-BA + 0.5 mg/L IBA + 0.5 mg/L Bar + 100 mg/L Tim) for regeneration. When the regenerated Basta-resistant seedlings grew to 1–2 cm, they were placed on rooting medium (1/2 MS + 10.0 g/L sucrose + 6.0 g/L agar powder + 0.2 mg/L IBA + 100 mg/L Tim) and cultured. Finally, the rooted plants were transplanted to a pre-treated mixed soil (2: 1: 1 loess : nutrient soil : perlite) after 2–3 d.

The DNA of Basta-resistant plants was extracted using the CTAB method for PCR identification.

qRT-PCR analysis

The *GhLAC1* expression level in transgenic tomato plants was assessed by real-time PCR using gene-specific primers, and the Clathrin adaptor complex (*CAC*) gene served as the internal reference (PCR primers *CAC*-F and *CAC*-R; Table 2). Real-time qRT-PCR was performed using a SYBR Green I Dye Kit (Applied Biosystems Inc., Foster, CA, USA) and the CFX Connect^{TM} Real-Time System (Applied Biosystems Inc.). Total RNA was isolated from the leaves of transgenic and control Micro-Tom, using the RNAiso Plus and Fruit-mate for RNA purification (TaKaRa, Dalian, China) and reverse-transcribed into cDNA by Reverse Transcriptase M-MLV (RNase H) (TaKaRa). The amplification cocktail was as follows: 3 μL template RNA, 1 μL oligo (dT)12–18 primer (50 μM) and 2 μL RNase free ddH_2O. This was placed at 70°C for 10 min and then on ice for 3 min. Afterward, 2 μL 5× M-MLV Buffer, 0.5 μL dNTP Mixture (10 m*M*), 0.25 μL RNase Inhibitor (40 U/μL), 0.25 μL RTase M-MLV (RNase H 200 U/μL) and RNase free ddH_2O were added up to 10 μL. The PCR cycles were 42°C for 1 h and 70°C for 15 min. Samples were then placed on ice for 3 min. The RT-PCR reaction

Table 1: Primer sequences for PCR amplification

Primer	Sequence
Gh-Forward	5 '-CCATTCCCAAAACCACAC-3'
Gh-Reverse	5 '-CGCTACTAAATACTTGCCAGA-3'

Table 2: Primer sequences for qPCR

Primer	Sequence
CAC-F	5 '-CCTCCGTTGTGATGTAACTGG-3'
CAC-R	5 '-ATTGGTGGAAAGTAACATCATCG-3'
GhLAC-F	5 '-AGGCTGTTGTCGGCATAG-3'
GhLAC-R	5 '-TCACTGTTGGACTTGGGATT-3'

system was as follows: 10 μL Power SYBR Green PCR Master, 0.2 μL forward primer, 0.2 μL reverse primer, 1 μL cDNA and 4.2 μL ddH_2O. The reaction conditions were 35 cycles of 98°C for 10 min, 58°C for 30 s and 72°C for 1 min, followed by a 12°C hold. All the experiments were repeated three times.

Analysis of the laccase activity

The laccase activity was determined using the ABTS method (Zhang 2007; Wang *et al.* 2008), and wild-type tomato was used as the control. Briefly, 0.1 g amples of the fifth leaf from the top of each tomato plant were taken. This was repeated three times. Samples were placed in liquid nitrogen and fully ground. The resulting powder was transferred into protein extraction buffer containing 25 mmol/L MOPS and 200 mmol/L $CaCl_2$, placed at 4°C for 4 min and centrifuged for 10 min at 10,000 ×g. The supernatant is the crude protein extract and it was added to 1 mL of the newly prepared 1 mmol/L ABTS solution. The absorbance A_1 was measured at 420 nm using a microplate reader. After 30 min of reaction at 30°C in a constant temperature water bath, the absorbance A_2 was measured at 420 nm. The laccase activity was calculated according to the following formula: enzyme activity = 1,000 × 0.1844 × (A_2−A_1) × 60 × 1,000 × dilution factor, the unit of enzyme activity is nmol/L/min, or U/g. A laccase activity unit (U) is defined as the amount of product produced by converting 1 nmol of ABTS substrate per gram of fresh tissue at 30°C for 1 min.

Analysis of pathogen resistance

The *in vitro* leaf inoculation method (Kovacs *et al*. 2013) was used. Wild-type plants were used as controls. Transgenic *GhLAC1*-expressing tomato having the same physiological state at the 5–6 leaf stage was used. After the leaf blade was cut from the petiole, the petiole was wrapped with sterilized cotton, and the cotton was moistened by adding an appropriate amount of sterile water. A 100-μL pipette was used to draw 50 μL of a *Botrytis spores* spore suspension at a 5 × 10^5 cfu/mL concentration. This was dropped on the front of the selected leaves and the leaves were cultured at 20°C.

Inoculation with sterile water was used as the blank control and the degree of gray mold infection was determined by measuring the diameters of leaf lesions after 5 d (Liu *et al.* 2016).

Determination of the lignin content

The lignin content was determined using the acetyl bromide method (Morrison 1972). Briefly, 1.0 g samples of tomato leaves from the top to the bottom of the fifth branch were taken, as was 1.0 g of a stem segment at 5 cm from the ground. Each sample was homogenized in 95% ethanol, centrifuged at 4,500 rpm for 5 min at room temperature, washed three times with 95% ethanol, and washed twice with a 1:2 (v/v) solution of ethanol: n-hexane. The precipitate was collected and dried at 60°C. Then, 2 mL of 25% bromoacetyl glacial acetic acid [1:3 (v/v) bromoacetyl: glacial acetic acid] solution was added to the dried precipitate. The dissolved precipitate was placed in a water bath at 70°C for 30 min and 0.9 mL of 2 mol/L NaOH solution was added. Then, 2 mL of glacial acetic acid and 0.1 mL of 7.5 mol/L hydroxylamine hydrochloride were added to the reaction solution to terminate the reaction and the volume was adjusted to 5 mL using glacial acetic acid. The sample was then centrifuge at 4,500 rpm for 5 min at room temperature. The supernatant was aspirated and the absorbance at 280 nm measured using a microplate reader. The lignin content is directly proportional to the optical density value at 280 nm and the relative absorbance of lignin per gram of fresh weight (FW) was expressed as the absorbance at 280 nm.

Electron microscope observations of tomato stem cross sections

The cross sections of the stem segments of wild-type and transgenic tomato plants were taken, and the samples were submerged in 2.5% glutaraldehyde fixative. After fixing for 12 h in a refrigerator at 4°C, the samples were thoroughly washed with buffer at room temperature. The buffers were a stepwise gradient concentration of ethanol as follows: 30, 50, 70, 80, 95 and 100% and each wash time was 15 to 20 min. Then, the sample was freeze dried and plated with using a Hitachi e-1010 ion sputtering apparatus. Then, the xylem cell wall morphology of the tomato stem section was observed using a Hitachi S3400 scanning electron microscope and photographed.

Statistical analysis

All experiments were repeated three times. The experimental data were statistically analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). In all these experiments, the quantitative differences between the compared data groups were statistically significant ($P < 0.005$). The $2^{-\Delta\Delta Ct}$ method was used to analyze qRT-PCR data.

Results

Identification of transgenic tomato

The plant expression vector PGM626-Act1-*GhLAC1* (Fig. 1) was transformed into *Agrobacterium*, and positive colonies were selected by colony PCR for tomato genetic transformation experiments (Fig. 2). The positive colonies had a target band of 399 bp (Fig. 3). Using wild-type and Basta-resistant tomato DNA as a template for PCR identification of transgenic plants, the results shown that the 399 bp specific band was amplified from transgenic tomato DNA, but not from wild-type (Fig. 4), indicating that *GhLAC1* was integrated into the tomato genome.

Relative expression of *GhLAC1* in transgenic tomato plants

The relative expression levels of *GhLAC1* were analyzed by quantitative reverse transcription real-time (RT)-PCR (Fig. 5). The expression of *GhLAC1* was highest in transgenic plant 12 (TP12), while the expression level in transgenic plant 9 (TP9) was much lower. The expression levels of *GhLAC1* in TP12 and transgenic plant 7 (TP7) were 4.71 and 4.54 times that of TP9, respectively. The difference in the relative expression between TP7 and TP9 is extremely.

Laccase activity in tomato

The laccase activities in three independent transgenic lines (TP7, TP9 and TP12) were determined and wild-type tomato was used as the control. The laccase activity levels of transgenic tomato plants overexpressing *GhLAC1* were exceedingly significantly higher than that of wild-type (Fig. 6). The laccase activities in TP9 (4,589.19 U/g), TP7 (6,773.50 U/g) and TP12 (6,879.96 U/g) were 1.47, 2.05 and 2.08 times that of the wild-type (3,307.52 U/g). The laccase activity of TP9 was significantly lower than those of TP7 and TP12, which had higher *GhLAC1* expression levels. The results supported that the increase in laccase activity in transgenic tomato plants is caused by the expression of *GhLAC1*, and they indicate that expressing the cotton laccase *LAC1* gene significantly increased the laccase activity in tomato.

Disease resistance assay with *Botrytis cinerea* Pers

To determine whether expressing the *GhLAC1* improved tomato disease resistance, the gray mold inoculation experiment (Fig. 7A-C) was performed on the leaves of wild-type tomato and the transgenic tomato line having the highest *GhLAC1* expression level (TP12). At 3 d after inoculation with *B. cinerea* Pers., the wild-type tomato leaves showed obvious round lesions, while the transgenic tomato leaves only showed spotted lesions (Fig. 7B). At 5 d after inoculation, the lesions of wild-type tomato leaves

Fig. 1: Diagram of expression vector of PGM626-Act1-*GhLAC1*.
RB: right border; LB: left border; Bar: Basta resistance marker; GUS: β-glucuronidase;
NOS: 3 'signal of nopaline synthase; Act1:*Actin1* promoter

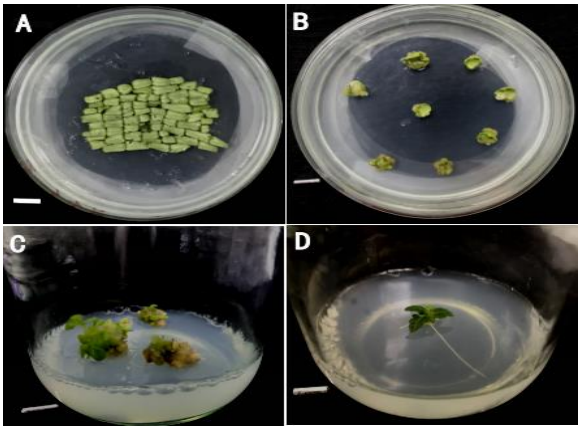

Fig. 2: The tomato genetic transformation process
A: co-culture; **B**: screening culture; **C**: differentiation; **D**: rooting

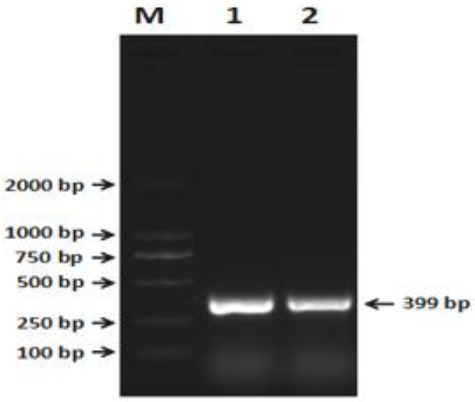

Fig. 3: Colony PCR for *Agrobacterium* strains harboring pGM626-Act1-*GhLAC1*
M: DNA marker DL 2000; 1-2: Positive colonies

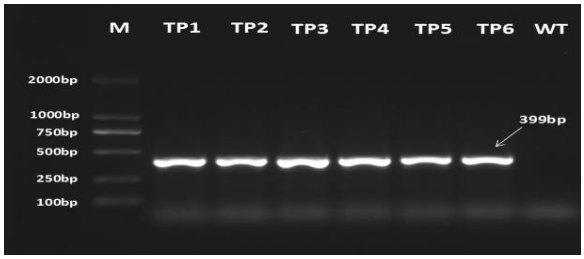

Fig. 4: PCR identification for transgenic tomato
M: DNA marker DL 2000; TP1-TP6: transgenic plants 1–6; WT: wild-type plant

increased significantly and started to spread to other uninfected leaflets; however, the lesions on leaves of transgenic tomato overexpressing *GhLAC1* were much smaller and did not spread (Fig. 7C). In addition, the diameters of the leaf lesions were statistically analyzed at 5 d

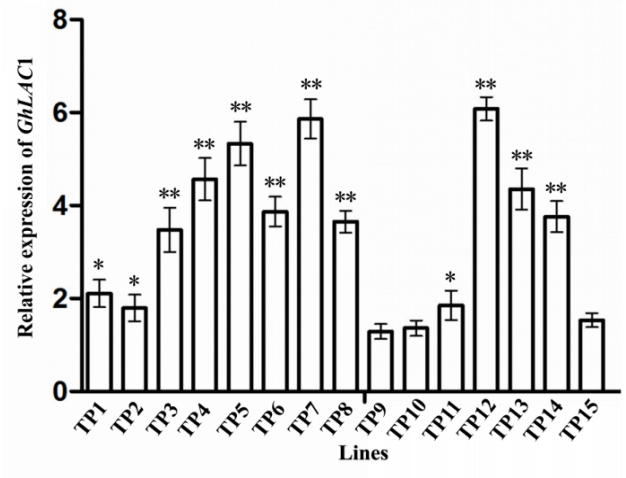

Fig. 5: Relative expression of *GhLAC1* in transgenic tomato plants
Error bars indicate standard error (SE); * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively

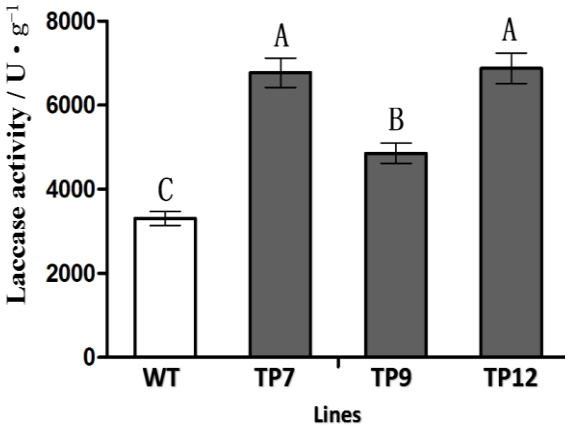

Fig. 6: Laccase activity in tomato
WT: wild-type plant; TP: transgenic *GhLAC1*-expressing plants
Error bars indicate standard error (SE); Letters on bars show whether the values are significant or not. Means having the same letter are not statistically significant ($P < 0.01$) according to least significant difference test

after inoculation (Fig. 8) and the average diameter of wild-type leaf lesions was 0.765 cm, while that of transgenic tomato leaves was 0.585 cm, which was an extremely significant 23.5% smaller than that of wild-type. This result indicated that expression of *GhLAC1* in tomato plants effectively increased tomato resistance to gray mold.

Lignin content and electron microscope observations of the tomato cell wall

A thicker cell wall can improve plant resistance to external stresses, and lignin is an important component of the cell wall. Consequently, the lignin content is closely correlated with disease resistance. The laccase gene is involved in the regulation of lignin synthesis; therefore, we hypothesized that the increased resistance of transgenic tomato expressing

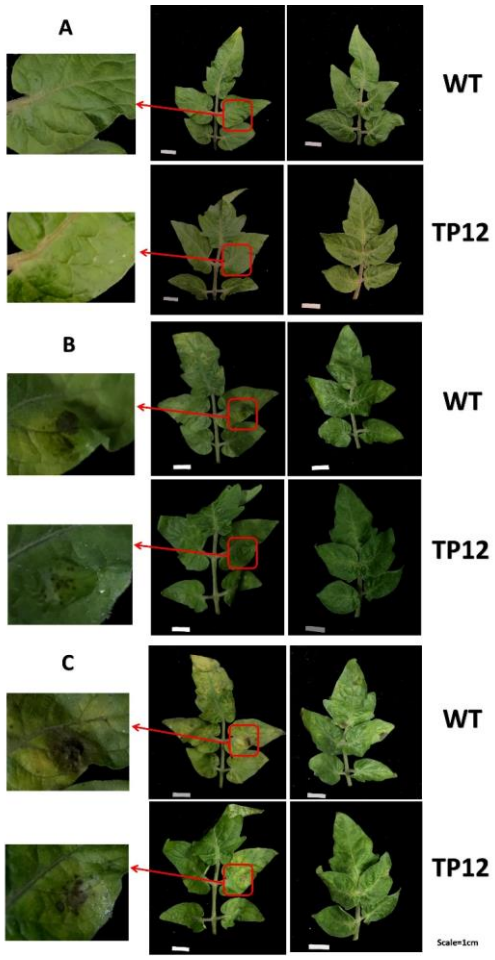

Fig. 7: The inoculation of wild-type and transgenic *GhLAC1*-expressing tomato plants leaves with *B. cinerea*
WT: wild-type plant; TP: transgenic *GhLAC1*-expressing plant; scale = 1 cm;
A: Not inoculated with *B. cinerea*; **B**: Three days after inoculation with *B. cinerea*; **C**: Five days after inoculation with *B. cinerea*

GhLAC1 to gray mold was caused by cell wall thickening. Therefore, the lignin contents of leaves and stems in wild-type and transgenic tomatoes were analyzed. The average lignin contents in the leaves and stems of wild-type tomato were 2.13 (OD_{280}/g FW) and 2.36 (OD_{280}/g FW), respectively, while those of transgenic tomato were 2.55 (OD_{280}/g FW) and 2.89 (OD_{280}/g FW), respectively, which were 19.7 and 22.5% higher than those of wild-type tomato, respectively (Fig. 9). The result suggested that the lignin contents in leaves and stems of transgenic tomato were significantly higher than those in wild-type, and the difference in the lignin contents of stems between wild-type and transgenic tomato plants was greater than that between leaves. Thus, over the expression of *GhLAC1* in tomato significantly increased the lignin content. Because the lignin content in the stem of the transgenic tomato was extremely significantly increased compared with the wild-type, further observations of the cross sections of wild-type and TP12 stems by scanning electron microscopy were performed.

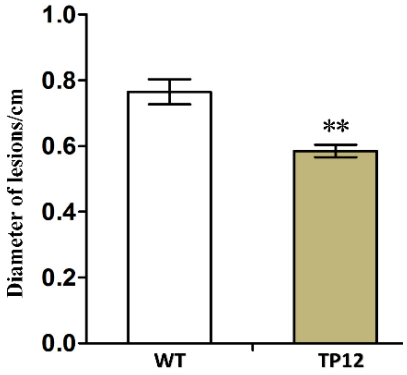

Fig. 8: Mean diameters of necrotic lesion in *B. cinerea*-inoculated tomato blades at 5 d after inoculation.
WT: wild-type plant; TP: transgenic *GhLAC1*-expressing plant
Error bars indicate standard error (SE); ** indicate significant differences at $P < 0.01$

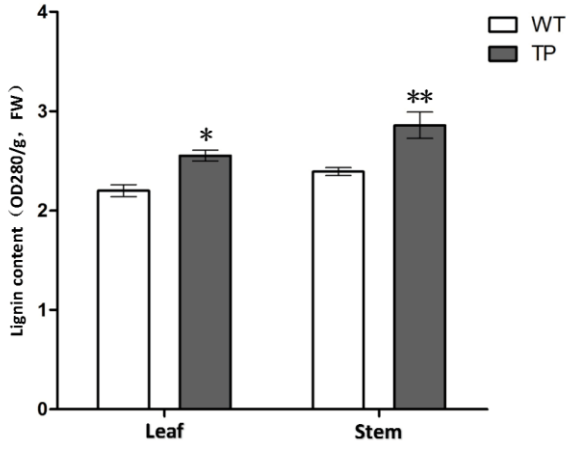

Fig. 9: Lignin contents in leaves and stems of wild-type and transgenic *GhLAC1*-expressing tomato plants.
Error bars indicate standard error (SE); * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively

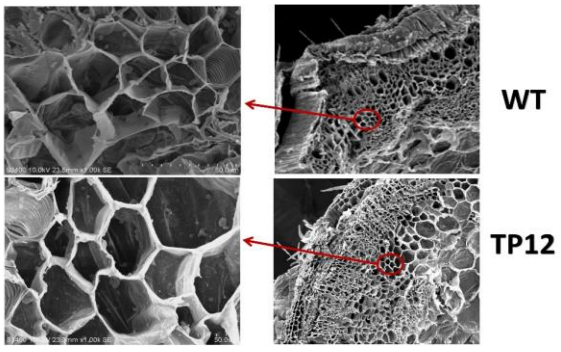

Fig. 10: Electron microscopic observations of stems of wild-type and transgenic *GhLAC1*-expressing tomato plants.
WT: wild-type plant; TP: transgenic *GhLAC1*-expressing plant; magnification 1,000×

The cell wall near the xylem of the transgenic tomato was thicker and more contoured than that of the wild-type (Fig. 10). Clearly, *GhLAC1* enhanced the cell wall of tomato. The correlation analysis between lignin content and lesion diameter revealed a high correlation. Higher the content of the lignin, the smaller was the lesion diameter (Table 3). Therefore, it provides evidence supporting the hypothesis that transgenic tomato overexpressing *GhLAC1* has a higher gray mold resistance than wild-type because the increase in the lignin content leads to the thickening of plant cell walls.

Table 3: Correlation between lignin and lesion diameter

	lignin content (OD_{280}/g, FW)	lesion diameter (cm)
WT	2.23 ± 0.05	0.81 ± 0.09
TP	2.49 ± 0.03	0.57 ± 0.03
Correlation coefficient	**-0.885 ***	

* indicate significant correlation at $P < 0.05$; TP: transgenic plants; WT: wild-type plant

Discussion

Lignin is a phenolic biopolymer derived from the phenylpropane pathway. It is an important component and can increase the strength of plant cell walls (Ding *et al.* 2016). There are three main types of monomers that make up lignin, coumarinol, coniferyl alcohol, and glucosinolate. They are oxidized and polymerized into p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin, respectively (Cao 2019). Laccase is thought to be involved in the polymerization of lignin monomers, which can oxidize lignin monomers and increase lignin deposition (Chou *et al.* 2018; Tobimatsu and Schuetz 2019). The laccase gene is involved in lignin synthesis in *Arabidopsis*, *Populus tomentosa*, and cotton (Cao 2016; Hu 2018; Chou *et al.* 2018). Here, the expression of the *GhLAC1* gene in tomato increased laccase activity, and the lignin contents in leaves and stems of transgenic tomato were significantly higher than those of wild-type. This is consistent with previous findings; therefore, we believe that an increase in laccase activity leads to an increase in the lignin content of transgenic plants. Lignin monomers are synthesized in the cytoplasm, secreted into the cell wall and then polymerized into lignin by oxidases, such as laccase and peroxidase, to increase the mechanical strength of the cell wall (Vanholme *et al.* 2010; Barros *et al.* 2015). Cheng *et al.* (2019) transformed the pear laccase gene *LAC1* into Arabidopsis and found that the transgenic plant had increased lignin content and thickened cell walls in interfascicular fibers and xylem cells. Scanning electron microscopy results in this study showed that the xylem cell walls of the transgenic plants were significantly thicker than those of the wild-type, and the outlines were more pronounced. We hypothesized that this was caused by the increase in lignin. Lignin can improve disease resistance in plants (Xia *et al.* 2015; Ma *et al.* 2017).

After a plant is infected with a fungus, lignin can induce plants to produce antitoxins or act as defense signaling molecules in the form of phenylpropane compounds (Mcfadden *et al.* 2001; Dixon *et al.* 2002; Naoumkina *et al.* 2010). Previous study found that the *CCoAOMT* of maize is related to the resistance of

pathogens, and it may affect plant resistance by participating in the phenylpropanin metabolism pathway (Yang *et al*. 2017). Yang *et al.* (2018) found that phenylalanine metabolism is involved in BcGs1-induced tomato defense responses to gray mold. Additionally, lignin improves plant disease resistance by strengthening the lignification of cell walls. Lignin provides a physical barrier that limits the colonization capabilities of pathogenic fungi, thereby increasing plant resistance (Bonello and Blodgett 2003; Zhang *et al*. 2017). In resistant cotton, the greater lignin content increases resistance to fungal diseases (Pomar *et al*. 2004). Gayoso *et al*. (2010) found that lignin synthesis plays a key role in the defense mechanism of tomato against Verticillium wilt. In wheat, a higher S-lignin content is regarded as a cell wall biochemical trait related to Fusarium resistance (Lionetti *et al.* 2015). High lignin content contribute to the basic defence response in tobacco (Ma *et al*. 2017). Here, we showed that the *GhLAC1* gene increased tomato resistance to gray mold, and we speculate that this may be correlated with the higher lignin content and cell wall enhancement in transgenic tomatoes.

In summary, we believe that the increased resistance of *GhLAC1*-expressing transgenic tomatoes to gray mold may result from the increase in lignin caused by the increase in laccase activity. Whether the mechanism of disease resistance results from the effects of phenylpropane compounds or the thickening of the cell wall caused by the enhancement of lignification remains to be studied further. In this study, the cotton laccase gene *GhLAC1* was genetically transformed into tomato for the first time and tomato plants with significant resistance to gray mold were obtained, which provides a new idea for tomato breeding against gray mold.

Conclusion

This study reported that expressing the *GhLAC1* gene in tomato increased its lignin content and resistance to gray mold. The results indicated that *GhLAC1* is a potential candidate gene for genetic engineering to develop crops with gray mold resistance which raises a possibility of improving plant defense. The detailed disease resistance mechanism of *GhLAC1*-expressing transgenic plants needs further research.

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References

Barros J, H Serk, I Granlund, E Pesquet (2015). The cell biology of lignification in higher plants. *Ann Bot* 115:1053–1074

Berthet S, N Demont-Caulet, B Pollet, P Bidzinski, L Cezard, P LeBris, N Borrega, J Hervé, E Blondet, S Balzergue, C Lapierre, L Jouanin (2011). Disruption of *LACCASE 4* and *17* results in tissue-specific alterations to lignification of *Arabidopsis thaliana* stems. *Plant Cell* 23:1124–1137

Bonello P, JT Blodgett (2003). *Pinus nigra-Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiol Mol Plant Pathol* 63:249–261

Cao CR (2019). Research progress on plant lignin synthesis and genetic engineering. *Mod Agric Sci Technol* 19:4–5

Cao S (2016). *Cloning and Functional Study of Lignin Formation Related Genes in Populus trichocarpa*. Circular Q945.1, Beijing Forestry University, College of Forestry, Beijing, China

Cesarino I, P AraJo, JLS Mayer (2013). Expression of *SofLAC*, a new laccase in sugarcane, restores lignin content but not S: G ratio of Arabidopsis *lac17* mutant. *J Exp Bot* 64:1769–1781

Cheng X, G Li, C Ma, M Abdullah, J Zhang, H Zhao, Q Jin, Y Cai, Y Lin (2019). Comprehensive genome-wide analysis of the pear (*Pyrus bretschneideri*) laccase gene (*PbLAC*) family and functional identification of *PbLAC1* involved in lignin biosynthesis. *PLoS One* 14; Article e0228183

Chou EY, M Schuetz, N Hoffmann, Y Watanabe, AL Samuels (2018). Distribution, mobility and anchoring of lignin-related oxidative enzymes in *Arabidopsis* secondary cell walls. *J Exp Bot* 69:1849–1859

Coego A (2005). An *Arabidopsis* homeodomain transcription factor, overexpressor of cationic peroxidase 3, mediates resistance to infection by necrotrophic pathogens. *Plant Cell* 17:2123–2137

Deng HM, K Shao, JH Liang, YT Chen, GX Yan (2017). Source of laccase and research progress on carriers for laccase immobilization. *Biotechnol Bull* 33:10–15

Ding X, CR Cao, PB Li, CC Wu, ML Cao, LL Yang (2016). Research progress on synthesis and regulation of plant lignin. *J Shanxi Agric Sci* 44:1406–1411

Dixon RA, L Achnine, P Kota, CJ Liu, MS Reddy, L Wang (2002). The phenylpropanoid pathway and plant defence-a genomics perspective. *Mol Plant Pathol* 3:371–390

Gayoso C, F Pomar, E Novo-Uzal, F Merino, D Martinez, O Ilarduya (2010). The Ve-mediated resistance response of the tomato to Verticillium dahliae involves H_2O_2, peroxidase and lignins and drives *PAL* gene expression. *BMC Plant Biol* 10; Article 232

Guo LX, D Xuan, DG Zhao (2016). Transgenic *EuCHIT1* tomato with *Eucommia ulmoides* chitinase gene enhances resistance to gray mold. *Chin J Plant Physiol* 52:703–714

Hu Q (2018). *Mechanism Analysis of Laccase GhLac1 Mediating Cotton Broad-spectrum Resistance*. Circular S435.62, Huazhong Agricultural University, College of Plant Science, Hubei, China

Hu Q, L Min, XY Yang, SX Jin, L Zhang, YY Li, YZ Ma, XW Qi, DQ Li, HB Liu, K Lindsey, LF Zhu, XL Zhang (2018). Laccase *GhLac1* modulates broad-spectrum biotic stress tolerance *via* DAMP-triggered immunity. *Plant Physiol* 176:1808–1823

Kiefer-Meyer MC, V Gomord, A O'Connell (1996). Cloning and sequence analysis of laccase-encoding cDNA clones from tobacco. *Gene* 178:205–207

Kovacs G, L Sagi, G Jacon, G Arinaitwe, JP Busogoro, E Thiry, H Strosse, R Swennen, S Remy (2013). Expression of a rice chitinase gene in transgenic banana ('Gros Michel', AAA genome group) confers resistance to black leaf streak disease. *Transg Res* 22:117–130

Li L, JC Steffens (2002). Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta* 215:239–247

Liang M, V Haroldsen, X Cai, Y Wu (2006). Expression of a putative laccase gene, *zmlac1*, in maize primary roots under stress. *Plant Cell Environ* 29:746–753

Lionetti V, A Giancaspro, E Fabri, SL Giove, N Reem, OA Zabotina, A Blanco, A Gadaleta, D Bellincampi (2015). Cell wall traits as potential resources to improve resistance of durum wheat against *Fusarium graminearum*. *BMC Plant Biol* 15:6-21

Liu CC, L Ming, XP Chu, HG Chen (2017a). Screening and identification of antagonistic bacteria from tomato *Botrytis cinerea*. *J Anhui Agric Sci* 45:26–27

Liu Q, L Luo, X Wang, Z Shen, L Zheng (2017b). Comprehensive analysis of rice laccase gene (*OsLAC*) family and ectopic expression of *OsLAC10* enhances tolerance to copper stress in *Arabidopsis*. *Intl J Mol Sci* 18; Article 209

Liu Z, Y Luan, J Li, Y Yin (2016). Expression of a tomato *MYB* gene in transgenic tobacco increases resistance to *Fusarium oxysporum* and *Botrytis cinerea*. *Eur J Plant Pathol* 144:607–617

Ma C, XY Zhou, Q Wang (2018). Research progress in biological control of *Botrytis cinerea*. *Hortic Seed* 2:61–62

Ma QH, HH Zhu, MY Qiao (2017). Contribution of both lignin content and sinapyl monomer to disease resistance in tobacco. *Plant Pathol* 67:642–650

Mcfadden HG, R Chapple, R Defeyter, E Dennis (2001). Expression of pathogenesis-related genes in cotton stems in response to infection by *Verticillium dahliae*. *Physiol Mol Plant Pathol* 58:119–131

Morrison IM (1972). A semi-micro method for the determination of lignin and its use in predicting the digestibility of forage crops. *J Sci Food Agric* 23:455–463

Naoumkina MA, Q Zhao, L Gallego-Giraldo, X Dai, PX Zhao, RA Dixon (2010). Genome-wide analysis of phenylpropanoid defence pathways. *Mol Plant Pathol* 11:829–846

Pan C, LT Lv, DG Zhao (2010). Establishment of *in vitro* flowering and seed setting system of transgenic tomato. *Mol Plant Breed* 8:818–821

Pomar F, M Novo, MA Bernal, F Merino, AR Barcelo (2004). Changes in stem lignins (monomer composition and crosslinking) and peroxidase are related with the maintenance of leaf photosynthetic integrity during Verticillium wilt in *Capsicum annuum*. *New Phytol* 163:111–123

Powell ALT, JV Kan, AT Have, J Visser, LC Greve, AB Bennett, JM Labavitch (2000). Transgenic expression of pear *PGIP* in tomato limits fungal colonization. *Mol Plant-Microb Interact* 13:942–950

Ranocha P, C Matthieu, C Simon, S Danoun, J Alain, B Alain-M, G Deborah (2002). Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. *Plant Physiol* 129:145–155

Tobimatsu Y, M Schuetz (2019). Lignin polymerization: how do plants manage the chemistry so well? *Curr Opin Biotechnol* 56:75–81

Vanholme R, B Demedts, K Morreel, J Ralph, W Boerjan (2010). Lignin biosynthesis and structure. *Plant Physiol* 153:895–905

Wang J, ML Zhu, ZM Wei (2008). Expression of cotton laccase gene in transgenic *Populus alba var. pyramidalis* and its effect on lignin synthesis. *Chin J Mol Cell Biol* 1:11–18

Wang Y, O Bouchabke-Coussa, P Lebris (2015). LACCASE5 is required for lignification of the *Brachypodium distachyon* Culm. *Plant Physiol* 168:192–204

Wang YH, ZM Yan, ZQ Jie, YN Feng, SY Cai (2016). The role of *SlCBL1* gene in the resistance to *Botrytis cinerea* of tomato. *Acta Bot Bor-Occid Sin* 36:2376–2384

Wang YL, P Zhao, CX Pei, QB Huang, H Jiang, HL Xi (2017). Research progress on laccase and its application. *Life Sci Instrum* 15:19–24

Wu LZ (2014). *Functional Analysis of Cotton Resistance to Verticillium Wilt-Related Gene GhLAC*. Circular S562, Agricultural University of Hebei, College of Agronomy, Baoding, China

Xia F, P Fan, MG Zhang (2015). Research progress on the role of secondary cell wall in plant disease resistance. *Hubei Agric Sci* 548:15–21

Yang C, Y Liang, D Qiu, H Zeng, J Yuan, X Yang (2018). Lignin metabolism involves *Botrytis cinerea* BcGs1-induced defense response in tomato. *BMC Plant Biol* 18; Article 103

Yang Q, Y He, M Kabahuma, T Chaya, A Kelly, E Borrego, YE Bian, F Kasmi, L Yang, P Teixeira, J Kolkman, R Nelson, M Kolomiets, LJ Dang, R Wisser, J Caplan, X Li, N Lauter, P Balint-Kurti (2017). A gene encoding maize caffeoyl-CoA O-methyltransferase confers quantitative resistance to multiple pathogens. *Nat Genet* 49:1364–1372

Zhang P (2007). Method for measuring laccase activity using ABTS as substrate. *Print Dyeing Auxil* 1:43–45

Zhang Y, LZ Wu, XF Wang, B Chen, J Zhao, J Cui, ZK Li, J Yang, LQ Wu, JH Wu, GY Zhang, ZY Ma (2019). The cotton laccase gene *GhLAC15* enhances Verticillium wilt resistance *via* an increase in defence-induced lignification and lignin components in the cell walls of plants. *Mol Plant Pathol* 20:309–322

Zhang Y, XF Wang, W Rong, J Yang, ZK Li, LQ Wu, GY Zhang, ZY Ma (2017). Histochemical analyses reveal that stronger intrinsic defenses in *Gossypium* barbadense than in *G. hirsutum* are associated with resistance to *Verticillium dahliae*. *Mol Plant Microb Interact* 30:984–996

Zhao Y, ZY Miao, Y Li, YJ Bai (2014). Research progress on tomato gray mold control. *Chin Plant Prot Guide* 34:21–29