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The mannose-binding lectin gene *CsMBL1* positively regulates resistance to citrus canker

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Abstract

Citrus canker is a serious bacterial disease caused by *Xanthomonas citri* (*Xcc*) subsp. *citri*. It is a significant threat to the citrus industry. Plants have several defense proteins, including mannose-binding lectins (MBL), which modulate innate immune responses by recognizing and binding to carbohydrates on microbial surfaces. However, the role of MBL in citrus canker resistance remains elusive. Previous transcriptomic analysis revealed that in the leaves of the citrus canker-resistant variety Kumquat (*Citrus japonica*), and the susceptible variety WanJincheng (*Citrus sinensis*) treated with *Xcc*, higher levels of *CsMBL1* were detected in the former compared to the latter, suggesting that *CsMBL1* is associated with resistance to citrus canker. Therefore, this study analyzed and identified a mannose-binding B-lectin gene, *CsMBL1*, and obtained the following results. Subcellular localization results indicated that *CsMBL1* is localized in the nucleus. Phytohormone induction experiments demonstrated that methyl jasmonate (MeJA) downregulated the expression of *CsMBL1* in Kumquat, while upregulating it in Wanjincheng. Moreover, its overexpression increased citrus canker resistance, while virus-induced gene silencing (VIGS) increased susceptibility. Biochemical analyses revealed increased reactive oxygen species (ROS) content, reduced peroxidase (POD) activity, and increased respiratory burst oxidase homologs (RBOHs) activity in *CsMBL1*-overexpressing plants. Additionally, the levels of abscisic acid (ABA) and jasmonic acid (JA) were diminished, while the salicylic acid (SA) level was elevated. These findings suggest that *CsMBL1* acts as a positive regulator of citrus canker resistance by modulating JA, ABA, SA, and ROS levels; therefore, *CsMBL1* can be targeted for modulation to develop canker-resistant citrus cultivars.

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Introduction

Citrus bacterial canker (CBC) is a plant bacterial disease caused by *Xanthomonas citri* (*Xcc*) subsp. *citri*, resulting in substantial losses to the global citrus industry[1]. CBC can spread through various pathways, making it difficult to prevent and control. The current disease control methods are ineffective; however, producing citrus canker-resistant citrus varieties through molecular breeding techniques can effectively control various diseases[2].

Plant lectins are a highly diverse group of carbohydrate-binding proteins that play roles in various biological processes. Based on phylogenetic analysis and domain structure, lectins are classified into 12 families, each comprising members derived from a single carbohydrate-binding-related domain[3]. The bulb-type mannose-specific binding lectin (B-lectins) belong to one of these families. These lectins can recognize and bind to mannose, modulating plant defense[4]. Mannose-binding lectins (MBLs) have been associated with plant growth. For instance, the G-type receptor-like kinase AsNIP43 interacts with the rhizobial effector nodulation outer protein P, which is essential for rhizobia-legume symbiosis[5]. Moreover, pollen development requires L-type lectin receptor kinase IV.2 (LECRK-IV.2)[6]. In *Brassica*, the S-locus receptor kinase containing a B-lectin domain modulates self-incompatibility responses[7].

The literature has validated that MBL plays a defensive role in many plant species. Furthermore, it is associated with salt stress in rice[8,9]. *OsJAC1* overexpression increases *Arabidopsis*'s resistance to ionizing radiation[10]. Moreover, in *Arabidopsis*, the MYB4-MAN3-mannose-MNB1 signaling cascade regulates cadmium tolerance *via* the GSH-dependent PC synthesis pathway[11]. MBLs are widespread in higher plants, and can interact with insect gastrointestinal systems, providing immunity against pests[12]. For instance, in transgenic crops, expressing snowdrop lectin GNA or feeding GNA provides resistance to lepidopteran pests[13,14]. In rice, GNA lectin gene overexpression increases resistance to the white-backed planthopper[15]. The upregulation of G-type lectin genes increases aphid resistance by inhibiting growth and fertility[16]. MBLs protect plants against pathogen attack by recognizing specific carbohydrates on pathogen surfaces and acting as a plant defense signaling mechanism. In rice, *OsCERK1* plays a key role in lipopolysaccharide (LPS)-induced immune responses[17]. For example, the B-lectin domain-containing gene *Pi-d2* confers resistance to rice blast[18]. *Arabidopsis* G-type lectin LORE, pepper G-type lectin genes *CaMBL1* and *CaGLP1* are involved in defense against *Xanthomonas campestris pv. vesicatoria* (*Xcv*)[19–21]. Moreover, in sunflowers, the jacalin-type MBL Helja interacts with fungal spore surfaces, inducing oxidative stress, and leading to cell death[22]. *FaMBL1*, an MBL gene, provides resistance to immature strawberry fruits against *Colletotrichum acutatum*[23].

Plants have various defense mechanisms, such as a complex immune system, to protect themselves against different pathogens[24]. Furthermore, several signaling molecules, such as reactive oxygen species (ROS), ethylene (ET), salicylic acid (SA), and jasmonic acid (JA), have been associated with plant defense networks[25]. The literature suggests that excessive ROS increases resistance against *Xcc*[26,27]. Moreover, JA and SA are key phytohormones that modulate plant immunity[28,29]. Further, plants confer resistance to citrus canker by regulating endogenous hormones such as JA and SA *via* multiple pathways[30]. Moreover, abscisic acid (ABA) has been found to negatively regulate resistance against pathogens, such as against *Botrytis cinerea* and *Pseudomonas syringae* in tomatoes[31,32]. Similarly, ET has a positive impact on plant disease resistance[33].

Various studies have investigated the association between MBL and biotic stress; however, its relationship with citrus canker remains unclear. Based on transcriptomic data from earlier infection experiments, using *Xcc* on citrus germplasms with contrasting resistance phenotypes, consistently higher expression of *CsMBL1* in the citrus canker-resistant variety Kumquat (*Citrus japonica*) was observed compared to the citrus canker-susceptible variety Wanjincheng (*Citrus sinensis*), across multiple time points. This expression pattern suggested a potential role of *CsMBL1* in CBC resistance, which became the focus of this study. To functionally characterize *CsMBL1*, genetic transformation and virus-induced gene silencing (VIGS) were performed in citrus. Integrated with biochemical assays, the present results reveal a mechanism by which *CsMBL1* contributes to CBC resistance. It was observed that *CsMBL1* contributes to the development of novel citrus canker-resistant citrus germplasm.

Materials and methods

Plants and bacteria

The Kumquat (*C. japonica*) and Wanjincheng (*C. sinensis*) citrus varieties were obtained from the National Citrus Germplasm Repository (Chongqing, China) and grown in a greenhouse at 28 °C for 3–5 months. Wanjincheng plants have greater susceptibility to citrus canker than Kumquat plants[34]. The *Xcc* variant, *XccYN1*, was isolated from infected citrus leaves and cultured at 28 °C in a peptone-yeast/malt extract medium augmented with 1.5% (w/v) D-glucose.

Bioinformatics analysis of *CsMBL1*

CsMBL1 coding sequence (CDS) was cloned and sequenced (Supplementary Table S1). This study acquired sequences from the CPBD database (http://citrus.hzau.edu.cn)[35,36]. Gene Structure Display Server (GSDS V2.0, http://gsds.gao-lab.org) was employed to predict gene structure[37]. Furthermore, functional domains were predicted using HMMER (http://hmmer.org)[38]. Moreover, MEGA X was utilized to construct a phylogenetic tree[39] using MBLs from different organisms (Supplementary Table S2).

Exogenous hormone-induced expression in citrus leaves

To conduct exogenous hormone assays, leaf discs (with a diameter of 7 mm) were immersed in different hormones (10 μmol/L ABA, 100 μmol/L methyl jasmonate [MeJA], 10 μmol/L SA, 10 μmol/L ET), and then sampled at 0, 6, 12, and 24 h post-treatment (hpt).

Overexpression in citrus

The complete CDS of *CsMBL1* was amplified using the following primers: $F_{OEC\text{-}CsMBL1}$ (containing KpnI restriction site: GGTACCATGA AGATGTCATCTTCCTCAGC) and $R_{OEC\text{-}CsMBL1}$ (containing 3 × flag tag and EcoRI restriction site sequence: GAATTCTTACTTATCATCATCAT CCTTGTAATCCTTATCATCATCATCCTTGTAATCCTTATCATCATCATCCTT GTAATCCTTGTTGGGAGCCTTTATGA). The amplified sequence was then inserted into the pGLNe vector comprising the CaMV 35S promoter, which was then transformed into *Agrobacterium tumefaciens* EHA105. Furthermore, the segments of Wanjincheng's shoot were transformed according to the method of Peng[2]. Transgenic plants were confirmed by PCR and GUS staining, and thereafter assessed for CsMBL1 expression levels with qRT-PCR. Plants with empty vectors were set as controls.

Subcellular localization

The recombinant plasmid *CsMBL1*-EAQGFP was constructed based on the stop codon lacking CDSs of *CsMBL1* using the following primer pair: $F_{SC\text{-}CsMBL1}$ (containing homologous arms and an AgeI restriction site:CTGCCCAAATTCGCGACCGGTATGAAGATGTCA TCTTCCTCAG), and $R_{SC\text{-}CsMBL1}$ (containing homologous arms and an AgeI restriction site: TCCTTTGCTAGTCATACCGGTCTTGTTGGGAGCCT TTAT). Then, the fragments were cloned into the EAQGFP vector by following the protocol of the In-Fusion cloning kit (TaKaRa, Japan). Subsequently, the constructed plasmids were transfected into *Nicotiana benthamiana* protoplasts. After 12 h, the GFP signals were measured using a laser scanning confocal microscope (Olympus FV3000, Japan).

Virus-induced gene silencing (VIGS)

For VIGS fragment (Supplementary Table S1) amplification, the following primer pair was employed: $F_{VIGS\text{-}CsMBL1}$ (containing homologous arms and an EcoRI restriction site: TTCTGTGAGTAAGGTTAC CGAATTCGGTAAATTTATTTGGCAGAGTTTTGATTATCCA)/$R_{VIGS\text{-}CsMBL1}$ (containing homologous arms and a BamHI restriction site: GACGC GTGAGCTCGGTACCGGATCCCGTGGGCCCCCAATCAA). The amplified region was incorporated into the TRV2 vector to produce TRV2-CsMBL1, and VIGS transformation was executed through infiltration with *Agrobacterium tumefaciens* EHA105[40]. After 30 d, Wanjincheng samples displayed green fluorescence under UV light, were pulverized in liquid nitrogen, and subsequently utilized for further analysis. The efficacy of VIGS silencing was assessed using qRT-PCR.

Citrus canker resistance analysis

Mature *CsMBL1* overexpressing leaves and VIGS plants were punctured 24 times (6 punctures/group). Then, each puncture site was injected with 1 μL of *XccYN1* (1×10^8 CFU·mL^{-1}), and leaves were placed on wet cotton at 28 °C for 10 d. The citrus canker disease on the leaves was assessed by evaluating the disease index (DI), and lesion area[2].

qRT-PCR analysis

Total RNA was extracted using the RNA Mini Preparation Kit (Aidlab, China), and reverse transcribed into cDNA using the PrimeScript Kit (Takara, Japan), and a mixture of 1 μg RNA, 2 μL PrimeScript buffer, and RNase-free water (to a total volume of 10 μL). The reaction conditions were set as follows: 37 °C for 45 min, 85 °C for 5 s, and 4 °C for 5 min. The qRT-PCR thermal cycling program was as follows: pre-denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, and 56 °C for 30 s. The reaction system (12 μL) contained cDNA (100 ng), primers (0.3 mmol/L), and PCR premix (6 μL). The relative gene expression was assessed *via* the $2^{-\Delta\Delta C_T}$ method[41]. The primers employed for qRT-PCR analysis were designed using the NCBI Primer BLAST tool and included: $F_{RT\text{-}CsMBL1}$ (GAGAGTTCGGGCCATACGTT) and $R_{RT\text{-}CsMBL1}$ (AACGCAAGCTGGAA TGGAGA). The primers for reference gene, *CsGAPDH* (CPBD ID: Cs5g06870)[42,43], were $F_{RT\text{-}GAPDH}$ (GCTTTCCGTGTACCCACTGT) and $R_{RT\text{-}GAPDH}$ (CTCTGACTCCGCCTTGATGG). The *C. sinensis* mRNA sequence was used as a standard for primer specificity evaluation.

Biochemical indices

Hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) concentrations were measured using commercial kits (Suzhou Keming Biotechnology, China). Furthermore, the expression levels of JA, SA, and ABA were determined *via* UPLC-MS/MS, while ET concentration

was measured using gas chromatography (GC). Peroxidase (POD), ascorbate peroxidase (APX), and respiratory burst oxidase homologs (RBOHs) activities were assessed using commercial kits (SinoBestBio, Shanghai, China).

Statistical analysis

Data were analyzed and visualized via GraphPad Prism V9.5 (San Diego, CA, USA), and depicted as means ± standard deviations (SDs). For intergroup comparison, a two-tailed *t*-test or Duncan's multiple range test followed by ANOVA was performed. $p < 0.05$ was considered statistically significant. qRT-PCR was performed in triplicate ($n = 3$).

Results

CsMBL1 encodes a mannose-binding B-lectin protein

CsMBL1 is present on chromosome 5 of sweet orange, and encodes a protein comprising 436 amino acids, with Bulb-type lectin, S-locus glycoprotein, and PAN domains. The *CsMBL1* gene is 1,308 bp long and comprises a single exon (Fig. 1a). Phylogenetic analysis revealed that *CsMBL1* is significantly associated with *Poncirus trifoliata* Ptrif.0003s1398.1.p. (Fig. 1b). The subcellular localization analysis revealed that CsMBL1 is primarily located in the nucleus (Fig. 1c).

Hormone treatments regulate *CsMBL1* expression in plants

In plants, phytohormones generally modulate the expression of disease-related proteins[44]. To investigate the role of *CsMBL1* in disease resistance signaling pathways, plants were treated with ABA, MeJA, SA, and ET, and then subjected to RT-PCR analysis to investigate *CsMBL1* expression. After exogenous ABA treatment, *CsMBL1* expression in both cultivars showed a consistent pattern of initial increase and then decrease (Fig. 2a). However, exogenous MeJA treatment induced contrasting *CsMBL1* expression patterns, exhibiting considerable downregulation in Kumquat and pronounced overexpression in Wanjincheng (Fig. 2b). In Wanjincheng, SA treatment initially significantly decreased *CsMBL1* expression, then increased, followed by a sharp decline at 24 h. Compared with Wanjincheng, *CsMBL1* expression indicated a more gradual decline in Kumquat (Fig. 2c). Moreover, ET induction resulted in distinct temporal expression patterns of *CsMBL1* between cultivars. In the Wanjincheng variety, *CsMBL1* expression was significantly upregulated within the first 12 h post-ET-treatment, followed by a sharp decline by 24 h, whereas in the Kumquat variety, *CsMBL1* expression was consistently elevated with no significant difference between 12 and 24 h timepoints (Fig. 2d). These results showed that *CsMBL1* expression induces phytohormones, suggesting its functional involvement in certain hormone-mediated disease resistance signaling pathways.

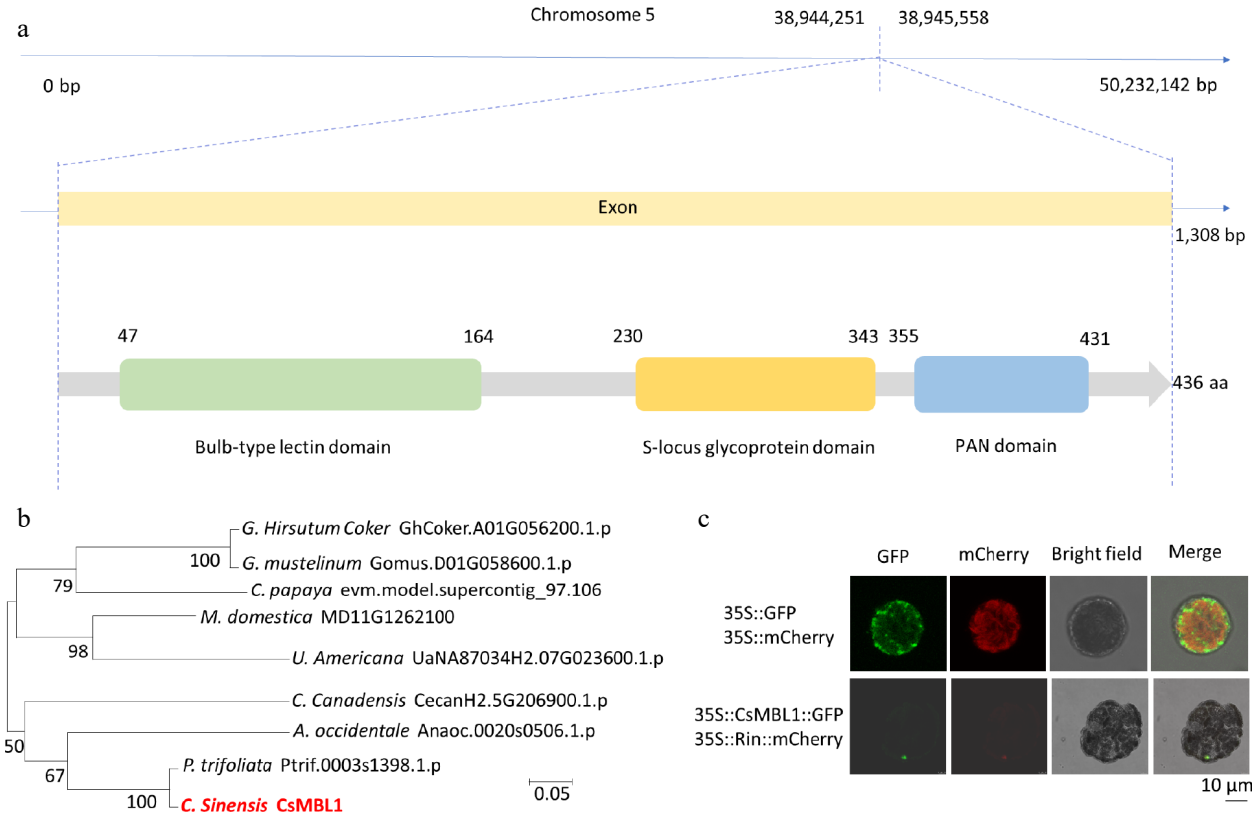

Fig. 1 Expression analysis and bioinformatics characterization of *CsMBL1*. (a) Chromosomal localization of *CsMBL1* was identified using CPDB (internal reference), and its exon-intron structure was determined using GSDS V2.0. *CsMBL1's* functional domains were identified using HMMER. (b) MEGA X (bootstrap: 500, Poisson model) was employed to construct a maximum likelihood phylogenetic tree of *CsMBL1* and its homologous proteins. Tree branches were proportional, with lengths representing the number of substitutions per site. Source species and protein IDs are indicated on the right side of the phylogenetic tree. (c) *CsMBL1* had a subcellular localization in *Nicotiana benthamiana* protoplasts. Images show GFP (green fluorescent protein), mCherry (red fluorescent protein), bright field, and merged channels. Scale bar = 10 μm.

CsMBL1 overexpression confers resistance against citrus canker

In this study, a *CsMBL1* overexpression plasmid was designed to assess its effects (Fig. 3a). The GUS staining indicated blue color on the leaf disc edge in three transgenic plants (PLGNe-CsMBL1-1, -2, -3), while no staining was observed in control plants (PLGNe) (Fig. 3b). PCR analysis detected the target fragment in the transgenic and positive control samples, but not in the control samples (Fig. 3c). Furthermore, qRT-PCR analysis validated *CsMBL1* overexpression in transgenic plants (Fig. 3d). Moreover, transgenic plants had normal growth rates (Fig. 3e), and reduced citrus canker symptoms compared to controls (Fig. 3f). Similarly, the lesion disease and area index were reduced by 11.5%–18.8% and 10.6%–15.4%, respectively (Fig. 3g and h). These data revealed that *CsMBL1* overexpression increases citrus canker resistance.

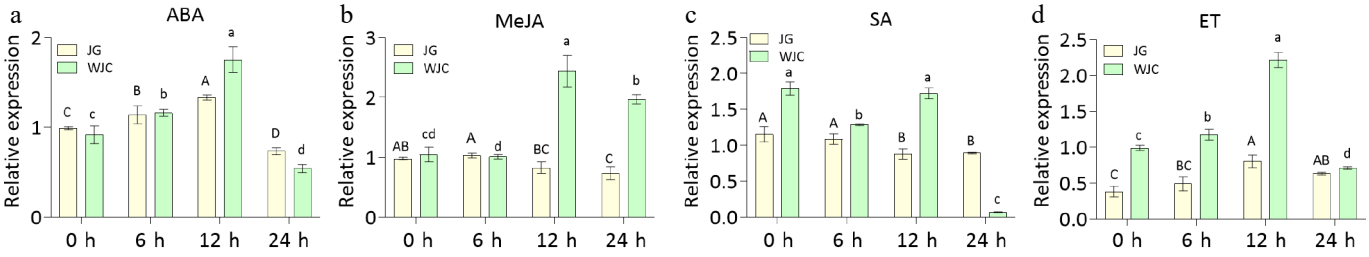

Fig. 2 Effects of phytohormone treatments on *CsMBL1* expression in plants. qRT-PCR analysis of *CsMBL1* expression in kumquat and Wanjincheng leaves following treatments with (a) ABA, (b) MeJA, (c) SA, (d) ET, and H_2O (control). CsGAPDH (CPDB ID: Cs_ont_5g044290) was used as the internal reference gene. Statistical comparisons were performed using ANOVA with Duncan's multiple range test. WJC: Wanjincheng; JG: Kumquat.

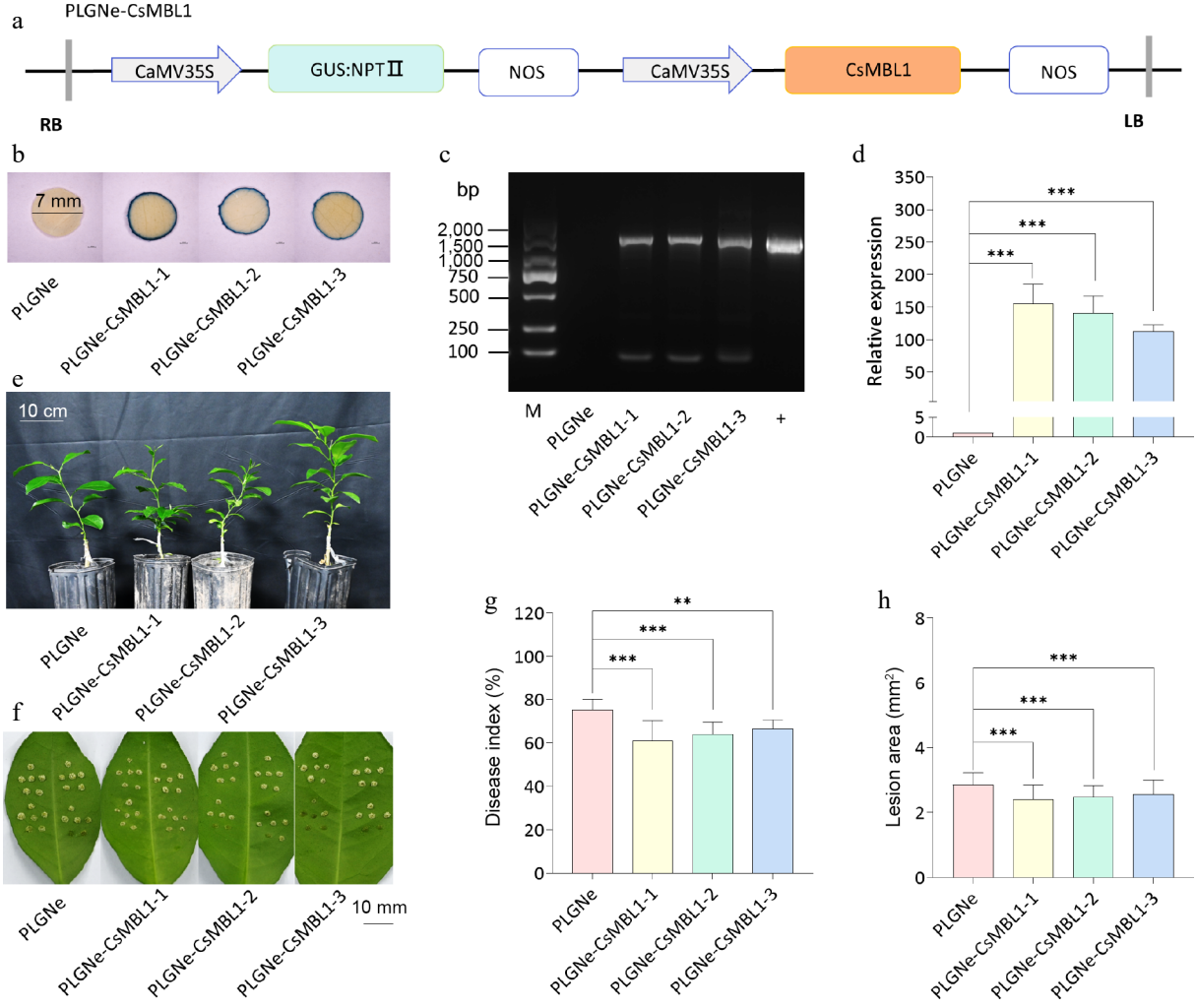

Fig. 3 Effects of *CsMBL1* overexpression on citrus canker resistance. (a) *CsMBL1* overexpression vector. (b) GUS staining results. Blue staining indicates transgenic plants. (c) PCR validation of transgenic plants. M: DNA marker; +: pLGNe-CsMBL1 plasmid. (d) The expression of *CsMBL1* relative to *CsGAPDH*. The control value was set to 1. Data are presented as mean ± SD of three replicates. (e) Phenotype of transgenic plants. (f) citrus canker symptoms in *Xcc*-infected leaves at 10 dpi. (g) Lesion disease, and (h) area index in *CsMBL1*-overexpressing plants at 10 dpi. (b)–(h) pLGNe-CsMBL1-1, -2, -3: *CsMBL1*-overexpressing materials, while pLGNe: control plants carrying the pLGNe vector. Data was statistically analyzed using Tukey's multiple range test ($p < 0.05$). NPTII: neomycin phosphotransferase II, NOS: NOS terminator, 35S: CaMV 35S promoter, GUS: β-glucuronidase and npt-linked coding gene, LB: left border, RB: right border, dpi: days post-infection.

VIGS of *CsMBL1* increases susceptibility to citrus canker

To evaluate the role of CsMBL1 in citrus canker resistance, *CsMBL1* was silenced in Wanjincheng plants. A *CsMBL1* VIGS plasmid was constructed using the TRV2 vector (Fig. 4a). Furthermore, *CsMBL1* silencing in VIGS plants was validated by PCR analysis using TRV1 and TRV2-specific primers (Fig. 4b). Compared to controls, VIGS plants had reduced relative expression of *CsMBL1* (Fig. 4c). Moreover, *Xcc*-infected VIGS plants had more severe citrus canker symptoms relative to control plants (Fig. 4d). Further, the calculated lesion areas (1.25, 1.18, and 1.32) (Fig. 4e) and disease index values (1.23, 1.17, and 1.33) of plants TRV2-CsMBL1-1, TRV2-CsMBL1-2, and TRV2-CsMBL1-3 were higher than that of controls (Fig. 4f). These results indicate that silencing CsMBL1 reduces resistance to citrus canker.

CsMBL1 overexpression modulates ROS homeostasis in plants

In plants, ROS are the first line of defense against pathogens[45]. Furthermore, H_2O_2 and $O_2^{\bullet-}$ serve as signaling molecules that regulate diverse cellular processes[46,47]. Here, it was observed that both H_2O_2 (Fig. 5a), and O_2^- (Fig. 5b) levels were increased in *CsMBL1*-overexpressing plants. RBOHs are primary producers of ROS in plants[48]. Moreover, POD and APX participate in ROS scavenging[49,50]. This study examined the activities of POD, RBOHs and APX, and revealed significant downregulation of POD activity (Fig. 5c), an evident increase in RBOH activity (Fig. 5d), while no significant difference in APX activity was observed compared to controls (Fig. 5e). These findings revealed that *CsMBL1* overexpression increases ROS levels through the coordinated regulation of POD and RBOH activities, therefore increasing resistance to *Xcc*.

CsMBL1 overexpression alters phytohormone levels

Phytohormones, including SA, JA, ABA, and ET, have been found to essentially modulate plant immunity[51–53]. To identify whether these hormones participate in *CsMBL1* overexpression-mediated resistance, PLGNe-CsMBL1-2 transgenic plants with superior resistance were selected to assess phytohormone levels. It was observed that *CsMBL1* overexpression significantly reduced ABA and JA levels (Fig. 6a and b), while increasing SA accumulation (Fig. 6c). There were no significant changes in ET content (Fig. 6d). These data suggest that *CsMBL1* overexpression increases citrus canker resistance in Wanjincheng by modulating hormone homeostasis, decreasing JA and ABA levels while increasing SA accumulation.

Discussion

The citrus canker is a destructive plant disease caused by *Xcc* and significantly adversely affects global citrus production[54]. Lectins are natural bioactive proteins with unique carbohydrate-binding

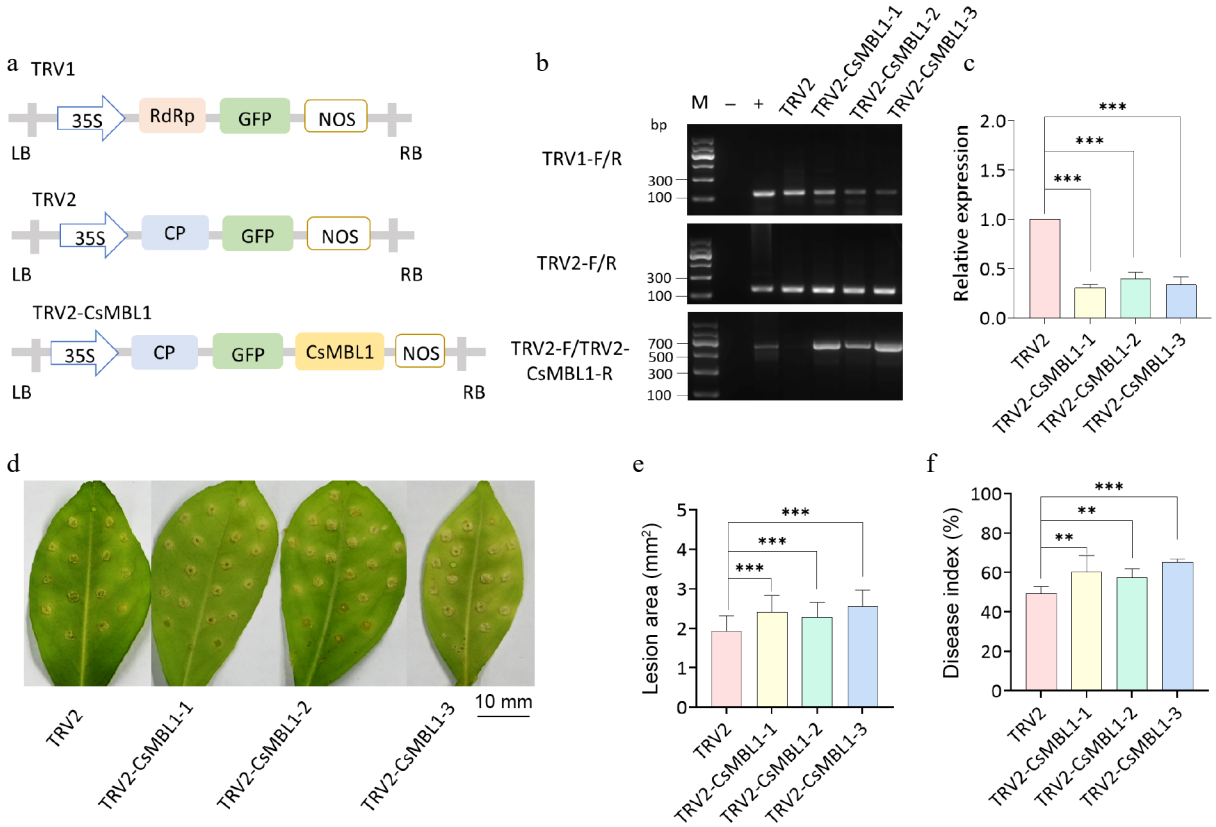

Fig. 4 Effect of *CsMBL1* silencing on citrus canker resistance. (a) Plasmid used for VIGS. (b) Successful transformation of VIGS plants was confirmed by PCR. M indicates DNA Marker, "–" represents ddH_2O, and "+" depicts plasmid. Primers used are shown on the left side of the gel electrophoresis image. (c) Relative *CsMBL1* expression was assessed by qRT-PCR using CsGAPDH as a control. The control value was set to 1, and the data represent the average of three biological replicates. (d) Disease symptoms, (e) lesion area, and (f) disease index of *Xcc*-infected VIGS plants at 10 dpi. (b)–(f) TRV2-CsMBL1-1, -2, and -3: *CsMBL1* VIGS plants; TRV2: control plants carrying the TRV2 vector (ANOVA with Tukey's multiple range test, $p < 0.05$). VIGS: virus-induced gene silencing (VIGS), 35S: Cauliflower mosaic virus 35S promoter, NOS: NOS terminator, GFP: green fluorescent protein, RdRp: RNA-dependent DNA polymerase, CP: coat protein, LB: left border, RB: right border.

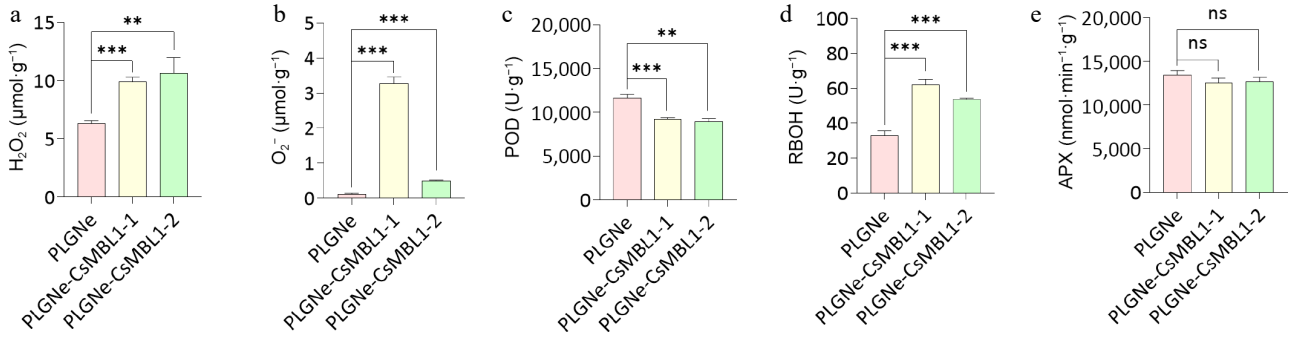

Fig. 5 Effects of *CsMBL1* overexpression on plant ROS levels. The contents of (a) H_2O_2, (b) O_2^-, and activities of (c) POD, (d) RBOH, and (e) APX were measured in *CsMBL1*-overexpressing plants. PLGNe-CsMBL1-1/-2: *CsMBL1*-overexpressing transgenic lines, PLGNe: Control plants carrying the empty PLGNe vector. Data were analyzed by ANOVA with Tukey's multiple range test, $p < 0.05$.

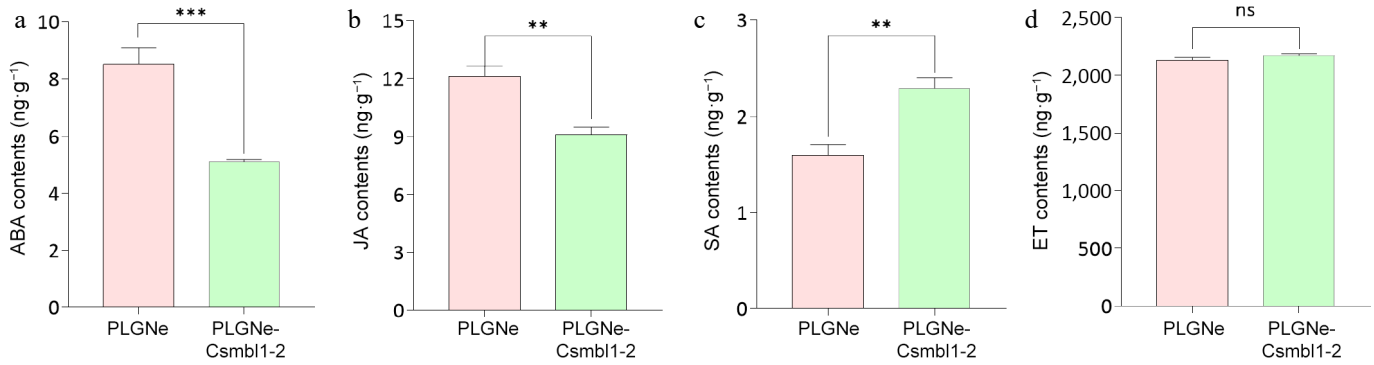

Fig. 6 Effects of *CsMBL1* overexpression on phytohormone levels. Concentrations of (a) ABA, (b) JA, (c) SA, and (d) ET were measured in *CsMBL1*-overexpressing plants. In (a)–(d): PLGNe-CsMBL1-2: *CsMBL1*-overexpressing plants. PLGNe: Control plants with an empty PLGNe vector. Data were statistically analyzed by ANOVA with Tukey's multiple range test ($p < 0.05$).

abilities. Furthermore, they have indicated antibacterial properties and facilitate immune responses[55]. Mannose-binding B-lectins specifically recognize mannose and function in innate immunity. The anti-pathogenic activity of MBLs has been observed in various species, including *Arabidopsis*, pepper, orchid, rice, and strawberry[18,20,56,57]. To evaluate whether citrus MBLs confer citrus canker resistance, *CsMBL1*, a citrus canker-induced gene encoding a B-lectin,wasanalyzed.Basedonthetranscriptomedata,weidentifiedand functionally characterized the differentially expressed gene *CsMBL1*. The data showed that *CsMBL1* overexpression alleviated canker symptoms, whereas VIGS exacerbated disease severity, suggesting that *CsMBL1* positively regulates citrus canker resistance.

The ROS signaling plays a pivotal role in plant innate immunity[58], and its accumulation promotes a plant adaptive response to stress conditions[46]. In sweet orange, the CsAP2-09-CsWRKY25-CsRBOH2 cascade confers *Xcc* resistance by modulating ROS homeostasis[27]. In rice, OsCERK1 downregulation inhibits the formation of ROS generated by LPS, which is a principal microbe-associated molecular pattern (MAMP) recognized by both plants and animals[17]. Here, it was found that CsMBL1-overexpressing plants had elevated H_2O_2 and O_2^- levels, as well as decreased POD and enhanced RBOHs activities. This ROS accumulation constitutes the fundamental plant defense mechanism[59], demonstrating that CsMBL1 mediates citrus canker resistance *via* ROS production.

Phytohormones, such as ABA, SA, JA, and ET, regulate plant defense mechanisms against biotic and abiotic stressors[60]. It has been observed that SA significantly regulates hypersensitive response and systemic acquired resistance[61], while ABA antagonizes defense-related hormones[62]. Moreover, JA and ET jointly modulate Botrytis cinerea-induced systemic resistance[63]. Furthermore, the relationship between ABA, SA, JA, ET, and resistance to citrus canker is well-documented in the literature[34,54,64]. In this study, the hormonal analyses revealed that CsMBL1 positively regulates SA while negatively affecting JA and ABA levels. MBLs are considered the regulators of the SA/JA pathways. For example, the overexpression of pepper CaMBL1 stimulates the production of defense genes and the formation of SA to counteract *Xanthomonas campestris pv vesicatoria* (*Xcv*) infection[20]. Moreover, *Arabidopsis* AtLEC is involved in JA/ET/chitin-triggered defenses[65], and strawberry *FaMBL1* overexpression decreases JA content[57].

These findings indicate that CsMBL1 acts as a modulator of ABA, JA, SA, and ROS homeostasis, thus increasing citrus canker resistance. Furthermore, *CsMBL1* expression is inducible by MeJA in Wanjincheng; however, its overexpression inhibits JA levels, indicating a self-regulatory loop that may modulate JA-mediated defenses. However, the present study on CsMBL1 remains insufficiently comprehensive. The relationships between CsMBL1 and phytohormones, ROS, and other signaling molecules require further investigation. Subsequent research could identify CsMBL1-interacting proteins and examine the expression of downstream defense-related genes to refine the regulatory network. These efforts would provide additional data to support the role of CsMBL1 in resistance against citrus bacterial canker. Additionally, Xcc-induced gene expression experiments can be conducted in both the citrus canker-resistant variety Kumquat (*C. japonica*), and citrus canker-susceptible variety Wanjincheng (*C. sinensis*). This would allow for the construction of a hypothetical model that integrates cultivar-specific data and functional data to explain the enhanced resistance to CBC observed in Kumquat and in *CsMBL1*-overexpressing 'Wanjincheng' plants.

Conclusions

In summary, this study demonstrated that CsMBL1 increases citrus canker resistance by coordinated regulation of defense components: (1) upregulating *CsMBL1* expression; (2) increasing SA and ROS (*via* RBOHs activation/POD suppression); and (3) reducing ABA/JA levels. Therefore, CsMBL1, as a positive regulator of citrus canker resistance, serves as a prospective molecular target for the development of canker-resistant citrus cultivars.

Author contributions

The authors confirm their contributions to the paper as follows: designed the experiments: Li Q; performed the experiments: Li M, Song Q, Lin D, Zhang M; analyzed the data: Li M, Song Q, Li Q, He Y; wrote the article: Li M, Li Q, He Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Conflict of interest

The authors declare that they have no conflict of interest.

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