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A simple and effective VIGS system facilitates the control of citrus canker by silencing *CsLOB1*

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Abstract

Virus-induced gene silencing (VIGS) is a powerful technology for molecular characterization of gene functions in plants and has the potential to prevent and control plant diseases. Unfortunately, VIGS in many woody perennials such as citrus are severely hindered because they have a long juvenile period and are recalcitrant to infection compared to herbaceous plants. Here, we report the development of a simple and effective VIGS system based on citrus leaf blotch virus (CLBV), a virus endemic in most citrus-producing countries but showing no symptoms in most citrus varieties. By *Agrobacterium*-mediated vacuum-infiltration (AVI), pCLBV201-su245 and pCLBV201-pds391 triggered efficient gene silencing (85%) and silencing phenotypes began to appear at 30 days post infiltration/inoculation (dpi), a similar period for achieving that on herbaceous plants. Moreover, the silencing phenotypes could still be observed four years post inoculation. Further, pCLBV201-lob369 was constructed to silence the gene *lateral organ boundaries1* (*lob1*), encoding a key factor for susceptibility to citrus canker that caused by *Xanthomonas citri* ssp. *citri* (*Xcc*). The resistance evaluation results showed that the lesion area, disease index, and bacterial content in the pCLBV201-lob369 treatment group were decreased by 64%, 14%, and 67%, respectively. This work provides a simple and effective VIGS system for citrus which has the potential to be used for diseases control.

Keywords Citrus leaf blotch virus (CLBV), Virus-induced gene silencing (VIGS), Citrus canker, Disease control

Background

Citrus is among the most important fruit crops in the world. However, the citrus industry faces numerous threats, in which citrus canker, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), is one of the most destructive diseases causing severe yield losses in most citrus-producing regions worldwide (Gottwald et al. 2002; Stover et al. 2014). Currently, the primary strategy for citrus canker control relies on the use of antibiotics or bactericides (Gottwald et al. 2002; Graham et al. 2004). However, potential disadvantages of these methods include high cost, risks to human health, and adverse environmental effects. At present, one way for survival of the citrus industry is to generate disease-resistant or tolerant varieties via transgenics. However, citrus trees are normally perennial woody crops with a long juvenile period, which make the genetic transformation labor-consuming and time-costing. Viral vectors can be deployed more quickly as an alternative approach to circumvent these issues. Viral vectors have been used to express valuable proteins in plants (Gleba et al. 2007; Lico et al. 2008), and can also be an advanced tool to identify the functions of host genes by reverse genetics. In addition, viral vectors can be used to modify the existing generation of trees. One such opportunity is to protect plants against diseases or to treat diseased plants. For example, a transient expression vector based on *Citrus tristeza virus* (CTV) was

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considered to be useful for controlling Huanglongbing (HLB) (Hajeri et al. 2014).

Over the last two decades, virus-induced gene silencing (VIGS) has emerged as an attractive tool to identify host gene function. VIGS exploits the hereditary post-transcriptional gene silencing mechanism in hosts, which is a defense system against pathogens naturally present in plants and other organisms (Robertson 2004). VIGS has many advantages such as simplicity, efficiency, and short cycle time, compared with traditional functional genomics research methods (Lu et al. 2017). Citrus leaf blotch virus (CLBV)-based vectors have been developed for either gene silencing or expression in citrus (Agüero et al. 2012). However, the viral vector cannot be transiently expressed in citrus leaves via *Agrobacterium* infiltration. Application of this technology to citrus is usually reliant on the use of tobacco (*Nicotiana* spp.) for virion enrichment, which is complicated and time-consuming. In addition, VIGS system based on CTV was established to control HLB (Hajeri et al. 2014). CTV genome is about 20 kb, and its genetic manipulation is very difficult. CTV can be transmitted by aphids, thus, it could be a biological risk if CTV was genetically manipulated (Shilts et al. 2020). Therefore, a simple and efficient VIGS system for citrus needs to be established.

CLBV is a member of the genus *Citrivirus*, family *Betaflexiviridae* (Martelli et al. 2007; Adams et al. 2012), and it has a single-stranded, positive-sense gRNA of 8747 nucleotides with three open reading frames (ORFs) and untranslated regions (UTRs) at the 5′ and 3′ ends of the gRNA (Galipienso et al. 2001; Vives et al. 2001). Potential advantages of CLBV-based vectors are: (i) CLBV causes a symptomless infection in most citrus species and cultivars (Galipienso et al. 2000). Therefore, phenotypic expression of gene silencing would not be masked. (ii) CLBV can invade meristematic regions (Agüero et al. 2013), thus those vectors would be appropriate for gene expression or silencing in non-phloem tissues including meristematic regions. (iii) Contrarily to CTV, CLBV is not transmitted by vectors and therefore it could be safely used in future field research.

In this study, a simple and efficient VIGS system for citrus was established based on CLBV by *Agrobacterium*-mediated vacuum-infiltration (AVI). The established VIGS system was evaluated and then used to silence the *lateral organ boundaries1* (*lob1*) gene to block the infection of *Xcc*, so as to control citrus canker.

Results

Construction of CLBV-based vector for gene silencing

In a previous work, a CLBV infectious clone named CLBV201 was constructed successfully. A unique *Sma*I restriction site was added just after the *CP* gene stop codon of CLBV201 to generate the vector pCLBV201 (Fig. 1). In order to evaluate the silencing capability, plants were inoculated with pCLBV201-gfp471 through

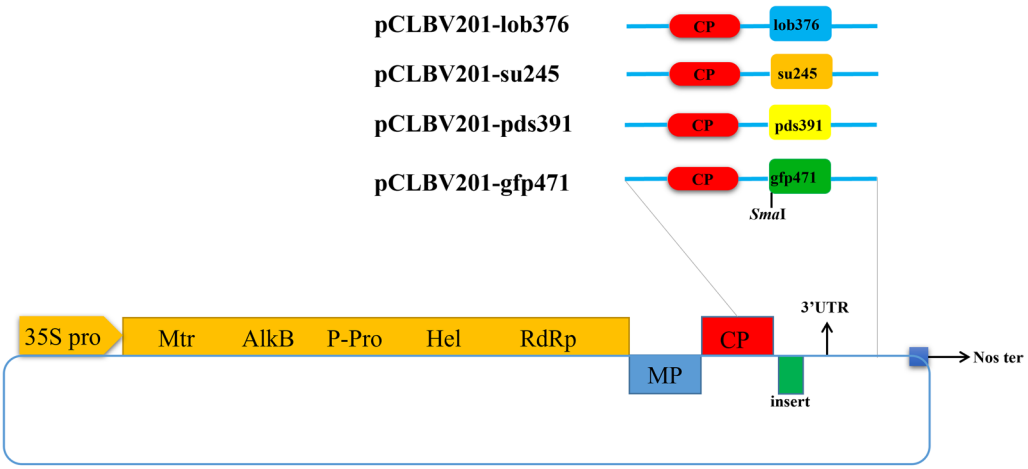

Fig. 1 Schematic representation of Citrus leaf blotch virus (CLBV)-based VIGS vector construction. Schematic representation of full-length infectious cDNA clones of CLBV with its open reading frames (ORF) placed between enhanced 35S promoter of cauliflower mosaic virus (CaMV) at the 5′ end, and nopaline synthase terminator (Nos ter) at the 3′ end in the ternary shuttle vector pCY. Introducing a unique restriction sites *Sma*I after coat protein (CP) gene. 471 bp fragment of green fluorescent protein (471 *gfp*) was cloned using unique restriction sites *Sma*I to generate pCLBV201-gfp471, similarly, 391 bp fragment of phytoene desaturase (391 *pds*), 245 bp fragment of sulfur (245 *su*), 376 bp fragment of LATERAL ORGAN BOUNDARIES 1 (376 *lob*) genes were cloned to generate pCLBV201-pds391, pCLBV201-su245, and pCLBV201-lob376, respectively. Mtr, methyltransferase motif; AlkB, AlkB-like peptidase; P-Pro, protease; Hel, helicase-like domain; RdRP, RNA-dependent RNA polymerase domain; MP, movement protein

Agrobacterium-mediated injection-infiltration (AII), while pCLBV201-wt was used as a control. GFP was monitored in the leaves, stems, and flowers by fluorescence observation under long wave UV (ultraviolet). The results showed that *N. benthamiana* transgenic line 16 c plants (containing a *gfp* gene) inoculated with pCLBV201-gfp471 began to appear green fluorescence quenching phenotypes at 7 dpi (Fig. 2a–f). The *gfp* silencing was further confirmed by RT-qPCR. The results showed that the *gfp* relative expression levels of pCLBV201-gfp471 treated group at 7 dpi, 20 dpi, 30 dpi, and 40 dpi decreased by 44%, 80%, 94%, and 99%, respectively, compared with the control group (Fig. 2g).

CLBV-induced gene silencing in citrus

Phytoene desaturase (PDS) and sulfur (SU) are two of the key rate-limiting enzymes in the carotenoid synthesis pathway and are often used as reporter genes to evaluate the success of VIGS. In the plants with carotenoids as the main pigment, carotenoids have a photoprotective effect. If the *pds* gene expression is silenced, the young leaves of the plants will show albino (photo-bleaching) phenotypes, with obvious phenotypic changes and easy observation and identification. If the *su* gene expression is silenced, the leaf will show chlorosis. In order to evaluate the efficacy of the CLBV-based VIGS system on citrus, pCLBV201-su245 and pCLBV201-pds391 vectors were constructed (Fig. 1). Eureka lemon seedings were inoculated with pCLBV201-su245 and pCLBV201-pds391 through AVI while pCLBV201-wt was used as a control. At 30 dpi, systemically infected leaves of *su* and *pds* silencing plants began to appear large yellow patches and photo-bleaching phenotypes, respectively (Fig. 3a, c), but there was no albino phenotype in the control (Fig. 3b, d). The expression levels of *su* and *pds* genes decreased by 86% and 88% (Fig. 3e, f), respectively, compared with the control, and corresponding contents of total chlorophylls and carotenoid decreased by 12% and 7% (Fig. 3g, h), respectively. Moreover, some new leaves of VIGS lemon plants still appeared silencing phenotypes and can be maintained for more than 12 months (Fig. 4). In addition, the *su* silenced citrus plants were grafted onto Ziyangxiangcheng (*Citrus junos* Sieb. ex Tanaka) rootstocks. Four years later photo-bleaching phenotypes were still observed on new leaves of the pCLBV201-su plants (Fig. 5a, b), but there was no albino phenotype in the control (Fig. 5c), and leaves showing the silencing phenotype had less than 65% *su* mRNA in comparison with their counterparts from plants inoculated with pCLBV201-wt (Fig. 5d), and the RT-PCR results showed that the expression of inserted fragment could be detected in the pCLBV201-245su plants, but not in pCLBV201-wt plants (Fig. 5e). These results indicated that this CLBV-based VIGS system was efficient and stable on citrus.

CLBV-based citrus plant-mediated RNAi in citrus canker

The results presented above suggested that CLBV vector could be successfully used as an efficient silencing vector. We designed a CLBV-VIGS vector, pCLBV201-lob369, to express a 369-nt sequence of the *CsLOB1* gene. Eureka lemon was inoculated with pCLBV201-lob369 through AVI into one-week-old citrus seedings and pCLBV201-wt was used as a control. We compared the relative expression level of *lob* gene of VIGS plant with untreated plants, and the result showed that the *lob* gene expression level was significantly reduced by 77% (Fig. 6a).

To characterize the canker resistance of VIGS plants, the plants with the expression of the *CsLOB1* gene silenced were evaluated for the resistance to *Xcc* by in vitro acupuncture. Disease development was determined at 10 dpi. In VIGS plants, eruption of pustules on the leaf surface was much slower than that of the wild type and, consequently, diseased lesions were markedly smaller than those in the wild type (Fig. 6b). These observations showed that silencing of the *CsLOB1* gene inhibited the development of pustules induced by *Xcc* infection. Statistical analysis showed that the number of bacteria on the leaves of VIGS plant was lower than that in the control group at the same dilution ratio (Fig. 6c). The lesion area on leaves of VIGS plants was reduced by 63% compared with that of the control (Fig. 6d). The disease severity of these lines was reduced by 69% compared with that of the wild type (Fig. 6e). The bacterial contents of VIGS plants significantly decreased by 67% compared with the wild type plants (Fig. 6f). These results indicate that *Xcc* growth was inhibited in the VIGS plants with the silenced gene *CsLOB1*.

Discussion

In this study, a simple and effective VIGS system based on CLBV was established. We use this system to silence the *su* and *pds* genes of citrus by AVI, and the results demonstrated that this system was easier and more rapid compared with traditional system. The common inoculation method on citrus was agroinfiltration with *N. benthamiana* for virion enrichment, then inoculated to citrus by stem slashing (Agüero et al. 2014). Recently, a highly efficient genetic transformation technology mediated by *A. rhizogenes* K599 in citrus was reported, which can bypass tissue culture, but the system was only useful in roots (Ma et al. 2022). In this study, *Agrobacterium* containing pCLBV201-su245 and pCLBV201-pds391 could be infiltrated into citrus seedlings directly by AVI. This inoculation method could by pass the complicated procedure of agroinfiltration with *N. benthamiana* for virion

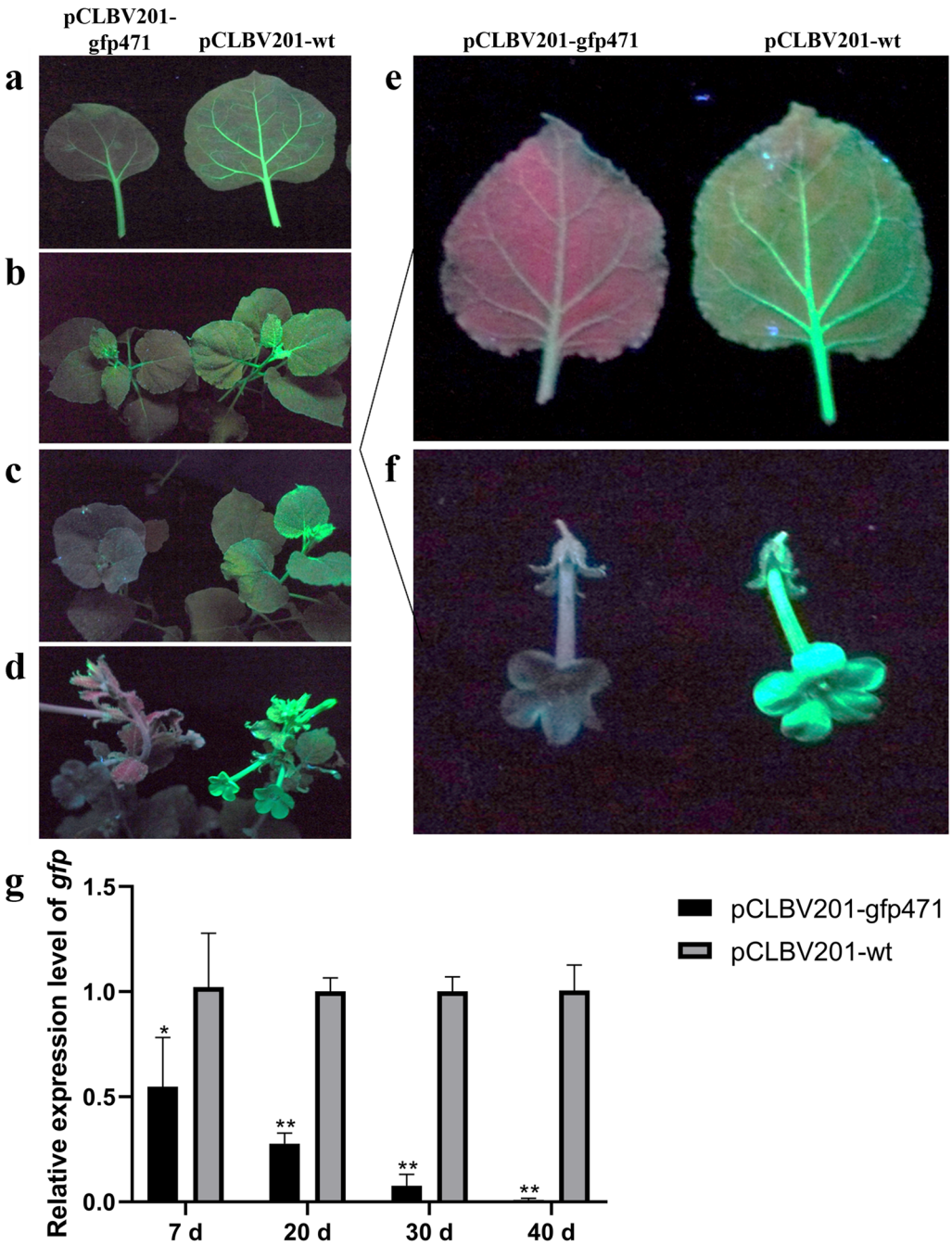

Fig. 2 Silencing phenotypes of *gfp* in *N. benthamiana* transgenic line 16 c by *Agrobacterium*-mediated injection-infiltration. **a** The seven days post inoculation (dpi), **b** 20 dpi, **c** 30 dpi, **d** 40 dpi, **e** silencing phenotypes in leaves. **f** The silencing phenotypes in flowers. **g** The relative expression level of the *gfp* gene of *N. benthamiana* transgenic line 16 c inoculated with pCLBV201-gfp471 or pCLBV201-wt by *Agrobacterium*-mediated injection-infiltration. 7 d, 7 dpi; 20 d, 20 dpi; 30 d, 30 dpi; 40 d, 40 dpi. The amount of *gfp* transcript was normalized to that of the *actin* gene in the same plants, the average expression in control plants infected with pCLBV201-wt was considered 1.0, and the normalized expression in plants infected with pCLBV201-gfp471 was relative to the average of the control plants. Bars represent standard deviation values and statistical significance was determined using student's *t*-test (*$P < 0.05$, **$P < 0.01$)

enrichment. Further, the silencing efficacy of the system was more effective than traditional system (Agüero et al. 2012, 2014). In this study, the *su* and *pds* genes silenced citrus appeared yellow and photobleaching phenotypes at 30 dpi, respectively, and the relative expression levels of *pds* and *su* genes were significantly reduced by 86%

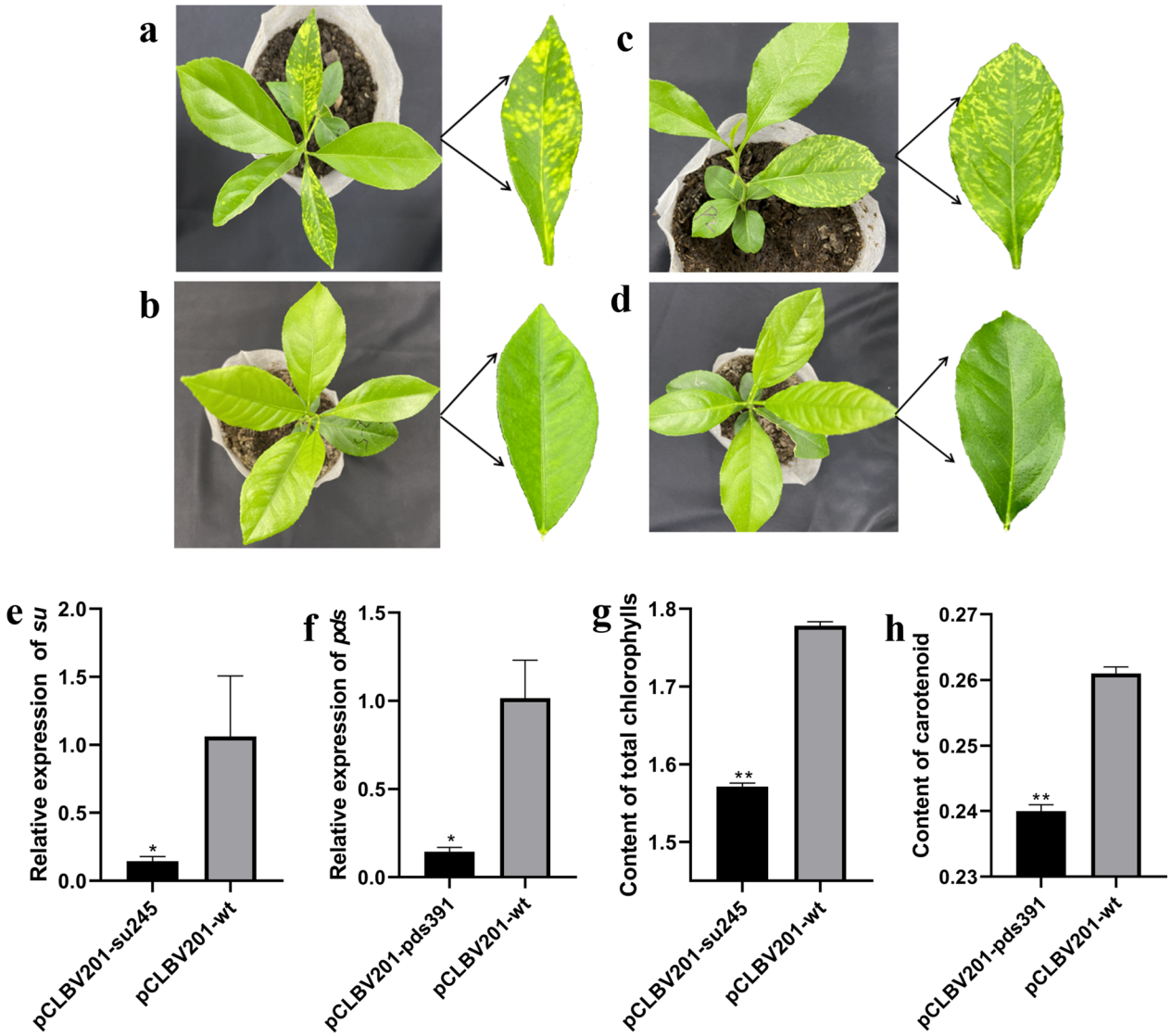

Fig. 3 Silencing phenotypes of *su* and *pds* in Eureka lemon by *Agrobacterium*-mediated vacuum-infiltration (AVI). **a** Eureka lemon plants inoculated with pCLBV201-su245 at 30 dpi. **c** Eureka lemon plants inoculated with pCLBV201-pds391 at 30 dpi. **b** and **d** Eureka lemon plants inoculated with pCLBV201-wt at 30 dpi. **e** and **f** The relative expression levels of the *su* and *pds* genes of Eureka lemon inoculated with pCLBV201-su245 and pCLBV201-pds391 or pCLBV201-wt at 30 dpi, respectively. For each gene, the amount of mRNA was normalized to that of the *actin* gene in the same plants. The average expression in control plants infected with pCLBV201-wt was considered 1.0, and the normalized expression in plants infected with the pCLBV201-su245 and pCLBV201-pds391 was relative to the average of the control plants. Bars represent standard deviation values and statistical significance was determined using student's *t*-test (*$P < 0.05$). **g** and **h** The contents of total chlorophylls and leaf carotenoid of Eureka lemon inoculated with pCLBV201-su245 and pCLBV201-pds391 or pCLBV201-wt at 30 dpi, respectively

and 88% (Fig. 3e, f), respectively. The VIGS effectiveness was assessed using insets of the *su* and *pds* genes in citrus, and the silencing efficacy was reduced by 50% and 70%, respectively (Agüero et al. 2012), and the silencing efficacy was lower than our CLBV-based VIGS system. Agüero et al. (2014) assessed the VIGS effectiveness based on some optimized silencing vectors, and the result showed a reduction of 74% (rough lemon) and 71% (sour orange) in *su* mRNA accumulation in comparison with the WT-inoculated control plants. Therefore, the CLBV-mediated VIGS system displays a high silencing efficiency on citrus by AVI.

VIGS is generally considered a transient assay system. However, the *su* and *pds* silencing phenotype in Eureka lemon remained for at least 12 months, and the leaves with silenced phenotype significantly increased. Moreover, the silencing phenotypes could still be observed and the *su* gene expression level decreased by 68% four years

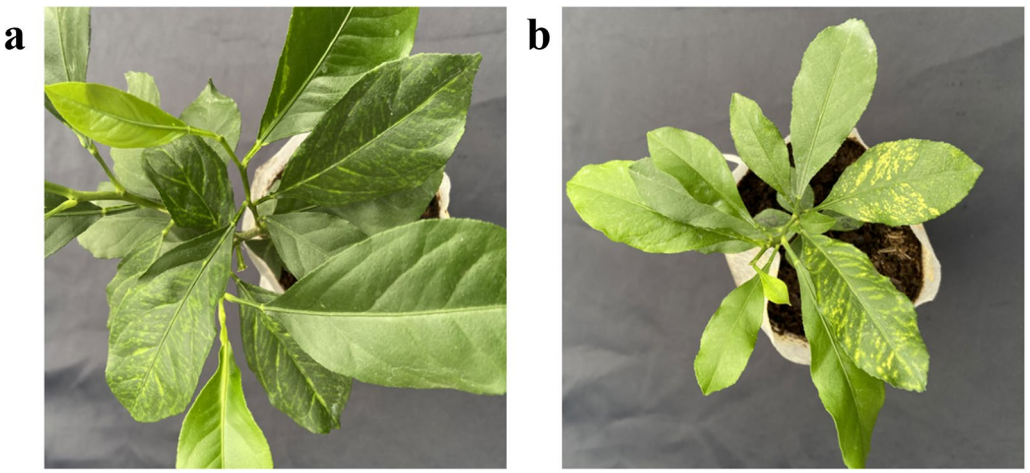

Fig. 4 Silencing phenotypes of *su* and *pds* in Eureka lemon by *Agrobacterium*-mediated vacuum-infiltration (AVI) at 12 months post inoculation. **a** Eureka lemon plants inoculated with pCLBV201-su245. **b** Eureka lemon plants inoculated with pCLBV201-pds391

after the silenced plants were grafted onto Ziyangxiangcheng rootstock. These results indicated that the established VIGS system could be performed stably in Eureka lemon.

Previous studies reported the successful use of CTV-based VIGS against *D. citri* (Hajeri et al. 2014), and *C*Las (Killiny 2020). These results demonstrated that CTV-based vector could be an interim solution in mitigating the spread of HLB disease in the field, which encourages us to apply CLBV-based VIGS system to control citrus canker. *CsLOB1*, the susceptibility gene for citrus canker, plays a critical role in promoting pathogen growth and erumpent pustule formation (Hu et al. 2014). Recently, Jia et al. (2016) reported that mutation of the coding region of *CsLOB1* in Duncan grapefruit (*Citrus paradisi*) successfully generated citrus canker-resistant plants. In 2017, the canker-resistant plants were obtained through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus (Peng et al. 2017). In this study, the *CsLOB1* gene was successfully silenced by pCLBV201-376lob, and its relative expression level was significantly reduced by 77% (Fig. 6a). Subsequently, the VIGS plants with the silenced *CsLOB1* gene were evaluated for resistance to *Xcc* by in vitro acupuncture. The results showed that the disease severity became mild obviously (Fig. 6b), and the number of bacteria groups was also significantly reduced (Fig. 6c). The lesions areas, disease index, and bacterial contents were reduced by 63%, 69%, and 67% (Fig. 6d–f), respectively. Indicating that silencing of the *CsLOB1* gene by VIGS had a certain control effect on *Xcc,* which provided a new idea for the control of citrus canker. Therefore, we presume that this CLBV-based VIGS system could be applied to control other diseases. For example, we can silence the genes associated with the morphological development of psyllids to control the spread of HLB (Hajeri et al. 2014). Traditional disease control measures mainly rely on resistant varieties, which is complicated and time-consuming. In this study, the CLBV-based VIGS system can bypass the transgenics process, without variety limitation, and this system appeared more rapid and stable than traditional control strategies. In addition, CLBV is not transmitted by vectors, and the inserted fragment will be lost finally. Therefore, the CLBV-VIGS system is safe for environment if used in disease control. In addition, CLBV can infect a wide range of plant species such as citrus, lemon, sweet cherry, peony, mulberry and kiwifruit (Guardo et al. 2007; Wang et al. 2016; Cao et al. 2017; Liu et al. 2019; Xuan et al. 2020). Therefore, the developed CLBV-based VIGS system may also contribute well to disease control and functional genomics investigations of some fruit trees such as kiwifruit and mulberry. The limitation of this system is that CLBV shows low accumulation and uneven distribution in some hosts (Galipienso et al. 2000, 2004; Ruiz-Ruiz et al. 2009), and is affected by temperature. In addition, CLBV can be transmitted by seed and grafting.

In summary, a simple and effective VIGS system based on CLBV was successfully established on citrus via AVI. CLBV-based vector was applied to silence the *CsLOB1* gene to control citrus canker, which provided an interim solution in mitigating citrus disease in the field.

Methods

Plant material and growth conditions

N. benthamiana transgenic line 16 c were grown in environmental chambers under a 16-h light/ 8-h dark cycle at 25°C. Eureka lemon (*Citrus limon* (L.) Burm.) was

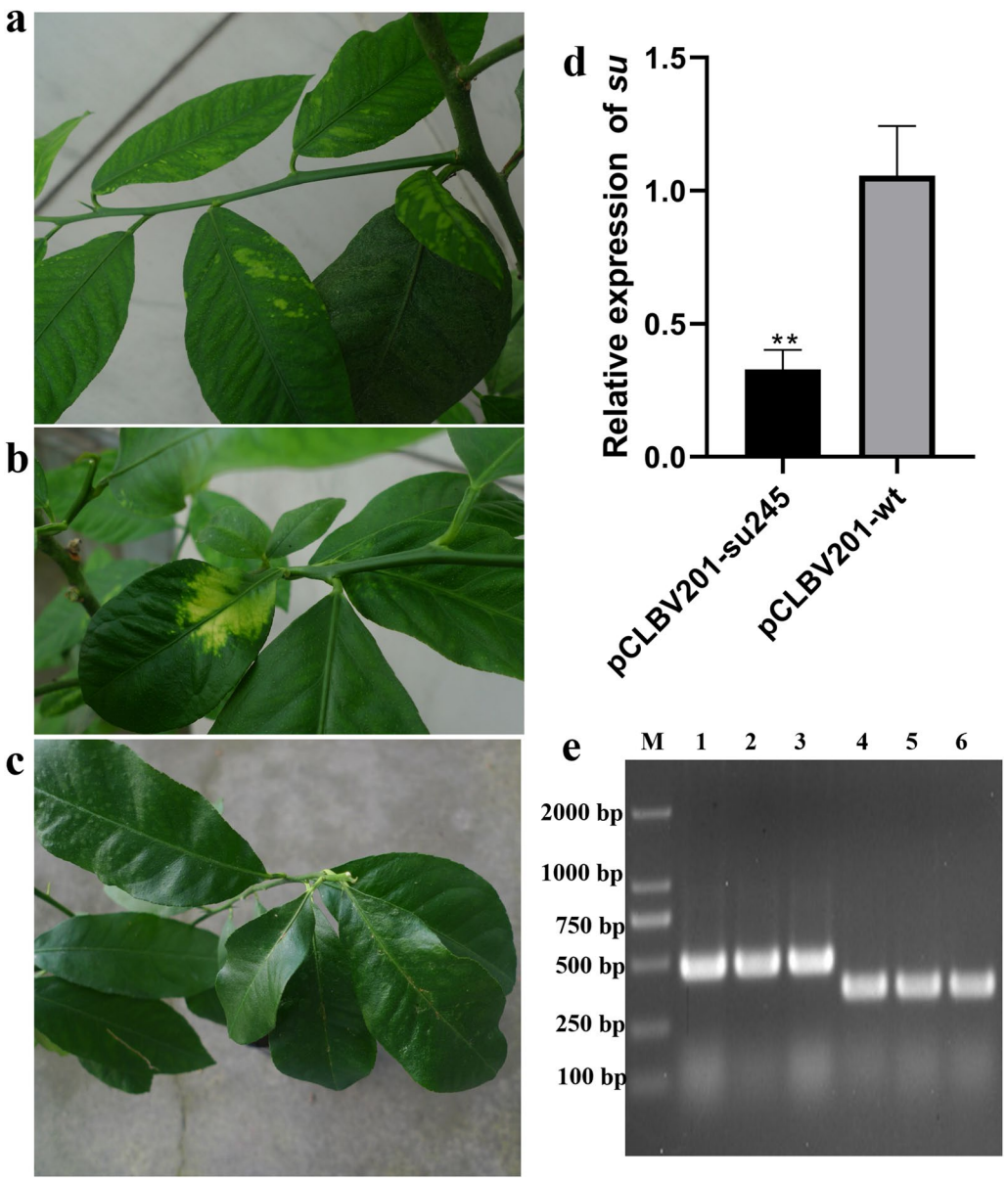

Fig. 5 Silencing phenotypes of *su* in Eureka lemon by *Agrobacterium*-mediated vacuum-infiltration (AVI) after grafted onto Ziyang xiangcheng (*Citrus junos* Sieb. ex TaKaRa) rootstock four years. **a** and **b** Eureka lemon plants inoculated with pCLBV201-su245. **c** Eureka lemon plants inoculated with pCLBV201-wt. **d** The relative expression levels of the *su* gene of Eureka lemon inoculated with pCLBV201-su245 or pCLBV201-wt after four years. The amount of mRNA was normalized to that of the *actin* gene in the same plants. The average expression in control plants infected with pCLBV201-wt was considered 1.0, and the normalized expression in plants infected with the pCLBV201-su245 was relative to the average of the control plants. Bars represent standard deviation values. **e** RT-PCR detection of inserted fragment of Eureka lemon. 1–3, pCLBV201-su245 inoculated plants; 4–6, pCLBV201-wt inoculated plants. M, DL2000 marker. Bars represent standard deviation values, statistical significance was determined using student's *t*-test (*$P < 0.05$, **$P < 0.01$)

grown as seedlings, and seeds were peeled from fresh fruits. After the removal of seed coats, mature seeds were surface-sterilized with 75% (v/v) ethanol for one min, 10% (v/v) Clorox containing 0.1% (v/v) Tween-20 for 30 min and then triple-rinsed with sterile distilled water. Fresh seeds germinated on a basal Murashige and Skoog (MS) medium supplemented with 50 g/L sucrose and solidified with 8 g/L agar. The pH was adjusted to 5.8 before autoclaving. The cultures were maintained in darkness at 28°C for one week.

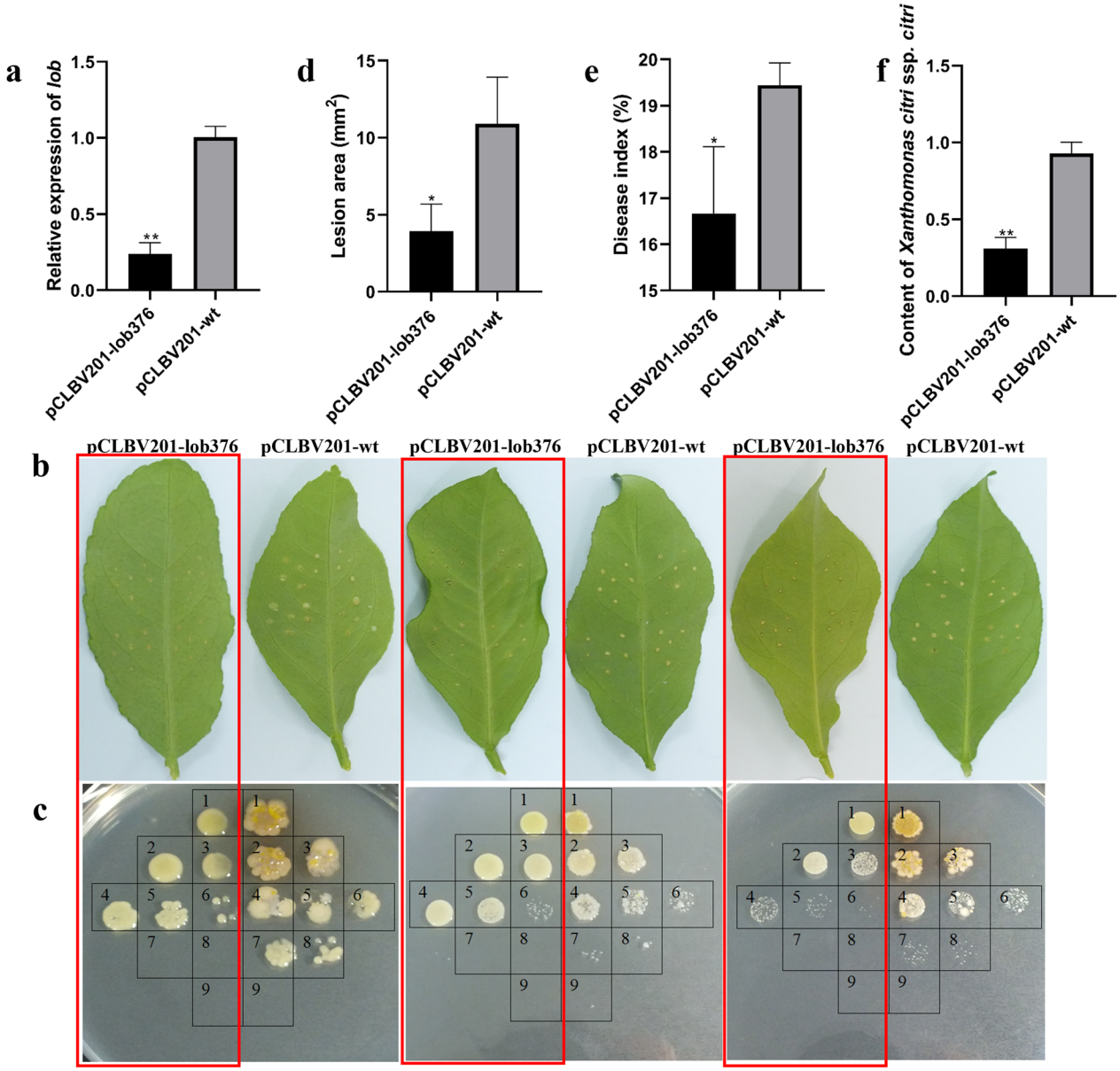

Fig. 6 Resistance evaluation of *Xanthomonas citri* ssp. *citri* (*Xcc*). **a** The expression level of the *CsLOB1* gene of VIGS plant. The amount of *lob* transcript was normalized to that of the *actin* gene in the same plants, the average expression in control plants infected with pCLBV201-wt was considered 1.0, and the normalized expression in plants infected with pCLBV201-lob369 was relative to the average of the control plants. **b** The symptoms of leaves inoculated with *Xcc,* circled in red color are the treated group that inoculated with pCLBV201-lob376, control group that inoculated with pCLBV201-wt. **c** The form of canker bacteria on a plate. 1–9: degree of dilution (1–9 represent: 1×5^{-1}, 1×5^{-2}, 1×5^{-3}, 1×5^{-4}, 1×5^{-5}, 1×5^{-6}, 1×5^{-7}, 1×5^{-8}, 1×5^{-9}). **d** The lesion area of leaves inoculated with *Xcc*. **e** The disease index of leaves inoculated with *Xcc*. **f** The content of leaves inoculated with *Xcc*. Bars represent standard deviation values, statistical significance was determined using student's *t*-test (*$P<0.05$, **$P<0.01$)

Total RNA extraction

Total RNA was extracted using the Trizol reagent (Ambion, America) and following the reagent's instructions. The 1st strand cDNA Synthesis SuperMix (Novoprotein, Japan) was used to generate the first-strand cDNA.

Vector construction

To construct VIGS vector pCLBV201, the infectious cDNA clone of CLBV201 in the yeast-*Escherichia coli-Agrobacterium tumefaciens* ternary shuttle vector pCY was used as base plasmid for engineering all the constructs used in this study (Cui et al. 2018). This plasmid contained CLBV genomic RNA between the

Table 1 List of primers used in this study

Primer name	Sequence (5′–3′)	Application
CP1125-F	tataacgtgtgactagtgtaaatagttagtACTTATCTAAATTAATCTAGATAGTGTACATCCA	Amplification of fragment CP1125
CP1125-R	CTACATTTCTAAGAGTTTTGCTTTGTT	
CP1080-F	aaaaacaaagcaaaactcttagaaatgtagcccgggTCCCGAATTCTGGCATG	Amplification of fragment CP1080
CP1080-R	actatagcagcggaggggttggatcaaagtACTTTGATCCCGAGGGGAACCCTG	
GFP471-F	tgccagaattcgggacccTCACTGGAGTTGTCCCAATT	Construction of pCLBV201-471gfp
GFP471-R	ctcttagaaatgtagcccAACTTGTGGCCGAGGATGTT	
SU245-F	tgccagaattcgggacccCGGGCTCTGTTTGCAGTTACC	Construction of pCLBV201-245su
SU245-R	ctcttagaaatgtagcccCGGGGAGTAAATCTACCAAGGACC	
PDS391-F	tgccagaattcgggacccAGCCTTTGCTTCAGCGTTTCTGAAAGTGCTTTC	Construction of pCLBV201-391pds
PDS391-R	ctcttagaaatgtagcccGTCTCATACCAGTTCCCGTCCCCATCTTTCC	
LOB376F	tgccagaattcgggaccc GAATGCAAACACAAAATTAAT	Construction of pCLBV201-376lob
LOB376R	ctcttagaaatgtagcccAACTTGT GGCCGAGGATGTT	
CLBV-F	CAATGCAGCAATTCCAACTGA	RT-PCR detection of CLBV infection
CLBV-R	GCGGTATCCCCTTTAGCATC	
Qgfp-F	AAAGCCAACTTCAAGACCC	RT-PCR detection of GFP mRNA
Qgfp-F	AAAGGGCAGATTGTGTGG	
Qsu-F	GGAGGAGAGAGCTCGATTTG	RT-PCR detection of SU mRNA
Qsu-R	GAGAGAACTCCTGGCTGAGG	
Qpds-F	TGTATTTGCCACACCTGTTG	RT-PCR detection of PDS mRNA
Qpds-R	TGGATGTTGATGACTGGAAC	
Qlob-F	AAAGCCAACTTCAAGACCC	RT-PCR detection of LOB mRNA
Qlob-F	AAAGGGCAGATTGTGTGG	
Xac F07	GAGTCGCCTACCGAGAAATCC	RT-PCR detection of xac mRNA
Xac R07	ACCACGGCAGGGTGAAGAC	
CitActin-F	CATCCCTCAGCACCTTCC	RT-PCR detection of citrus Actin mRNA
CitActin-R	CCAACCTTAGCACTTCTCC	
Tactin-F	AAGGGATGCGAGGATGGA	RT-PCR detection of tobacco Actin mRNA
Tactin-R	CAAGGAAATCACCGCTTTGG	

The lower case part is 18 bp homologous arm sequence

duplicated 35S promoter of cauliflower mosaic virus in the 5′ end, a ribozyme sequence of Subterranean clover mottle virus satellite RNA at the 3′ end. Unique restriction sites, *Sma*I were introduced after coat protein gene stop codon of CLBV, to ligate the inserts under coat protein (CP) sub-genomic RNA controller element (CE) between CP and 3′-untranslated regions. Then, to generate pCLBV201-gfp471, pCLBV201-su245, pCLBV201-pds391, and pCLBV201-lob376, the pCLBV201 vector was digested with restriction enzyme *Sma*I, and the fragment 471GFP of the green fluorescent protein (*gfp*) gene, fragments 245SU, 391PDS, and 376LOB of the sulfur (*su*), phytoene desaturase (*pds*), and lateral organ boundaries1 (*CsLOB1)* genes were inserted into the digested pCLBV201 vector to obtain the pCLBV201-gfp471, pCLBV201-su245, pCLBV201-pds391, and pCLBV201-lob376 constructs, respectively (Table 1).

Agrobacterium preparation and inoculation

pCLBV201-su245, pCLBV201-pds391, and pCLBV201-lob376 constructs were inoculated onto Eureka lemon seedlings by AVI that was performed as previously described (Kurth et al. 2012; Wang et al. 2017; Cui et al. 2018). Briefly, *A. tumefaciens* strain GV3101 harboring pCLBV201-gfp471, pCLBV201-su245, pCLBV201-pds391, and pCLBV201-lob376 or RNA silencing suppressor were grown at 28°C for 24–36 h in solid LB (Luria–Bertani) medium containing antibiotics (20 mg/L rifampicin, and 50 mg/L kanamycin). Then, the *A. tumefaciens* was transferred to LB liquid medium containing the corresponding antibiotics and cultivated overnight in a 28℃ shaker. Then, *Agrobacterium* cells were harvested and resuspended in infiltration buffer (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 200 μM acetosyringone) with optical density at OD_{600} of 1.0 for the vector-containing bacterium and 0.3 for RNA silencing

suppressor HC-Pro-P19-γb (Wang et al. 2015). One week old citrus seedlings were then submerged in the bacterial suspensions for 30 s in a vacuum chamber, followed by a quick pressure release as described (Wang et al. 2017). pCLBV201-gfp471 agroinfiltration was performed with 1 mL syringes without needle on plantlets 4–6-leaf stage. Plants infiltrated with *Agrobacterium* containing pCLBV201-wt and the HC-Pro-P19-γb expression plasmid were served as mock-inoculation control (Mock). Inoculated plants were grown in a growth chamber with a 16 h light at 25°C and 8 h dark at 22°C.

Assay of resistance to citrus canker

To evaluate the disease resistance of VIGS plants to *Xcc*, the in vitro assay was conducted as described by Peng et al. (2015). Three leaves of pCLBV201-lob376 plants and pCLBV201-WT plants were tested, respectively. Per leaf were made with a pin (0.5 mm in diameter), and per leaf including four infected sites that comprising six punctures. One microliter *Xcc* bacterial suspension (1×10^5 CFU/mL) was applied to each puncture site. Photographs were taken at 10 days post inoculation (dpi). The lesion area was assessed with ImageJ 2.0 software. Then two groups of diseased spots were collected and grinded with 500 μL ddH_2O, then diluted five folds gradiently with sterilized ddH_2O (1–9 represent: 1×5^{-1}, 1×5^{-2}, 1×5^{-3}, 1×5^{-4}, 1×5^{-5}, 1×5^{-6}, 1×5^{-7}, 1×5^{-8}, and 1×5^{-9} respectively). Five microliter of lapping solution and diluted lapping solution were sequentially dropped on LB plate (without antibiotics) and cultured inversely at 28°C for two days. The disease intensity of per leaf was based on 24 punctures using the following rating index: 0, < 0.25 mm^2 (the size of the inoculating pin); 1, 0.25–0.75 mm^2; 3, 0.75–1.25 mm^2; 5, 1.25–1.75 mm^2; 7, > 1.75 mm^2. The disease index (DI), indicating the level of resistance to *Xcc*, was calculated with the formula:

$$DI = \Sigma[\text{numberof each index}\times\text{the corresponding index}]/(\text{number of lesions}\times\text{the max index})\times100$$

Two groups of diseased spots were collected and the DNA extracted to detect the accumulation level of *Xcc*.

RT-PCR detection and RT-qPCR analysis

The sequences inserted into the CLBV viral vectors were detected by conventional RT-PCR with the primer pairs CLBV-F/GFP471-R, CLBV-F/SU245-R and CLBV-F/PDS-R flanking the three insertion sites. The DNA synthesized was analyzed by 1% agarose gel electrophoresis and GelRed-staining.

To investigate the expression levels of the *gfp*, *su*, and *pds* genes in silenced citrus and *N. benthamiana* transgenic line 16 c, leaves of these plants exhibiting the silencing phenotype were collected from the silenced seedlings (treated with pCLBV201-gfp471, pCLBV201-su245, and pCLBV201-pds391). Leaves were also harvested from mock-treated (treated with pCLBV201-wt) at 90 dpi. Real-time PCR was performed using SYBR Premix ExTAq with cDNA from the leaves of the mock-treated and silenced seedlings. The tobacco *actin* and citrus *actin* used as the reference genes were amplified using primers Tactin-F/R and CitActin-F/R, respectively. *Actin* was used as an internal control to normalize for PCR efficiency. The relative expression levels of *gfp*, *su*, and *pds* were analyzed using the $2^{-\Delta\Delta CT}$ method and its expression values were calculated relative to the highest expression value, which was set as 1.0 (CK). Data were analyzed by analysis of variance with the SPSS software. Error bars represent ± SE of data from three independent experiments. Different letters indicate significant differences at $P \leq 0.05$ (Table 1).

Measurement of chlorophyll and carotenoid contents

The leaves of mock and treated plants at the one and two months post inoculation were collected for the measurement of chlorophyll and carotenoid contents. The method is employed according to the kit instructions (#YX-W-700) (Sinobestbio, Shanghai, China). Approximately 100 mg of leaves were cut into pieces and submerged in the 10 mL of mixed extract (acetone: anhydrous ethanol: distilled water = 4.5: 4.5: 1) for 18–24 h in the dark to extract the chlorophyll and carotenoid. After centrifugation at 5000 *g* for 10 min, the absorbance of the extract was measured at 663 nm (chla), 645 nm (chlb), and 470 nm (carotenoid) by a UV-vis spectrophotometer. Chlorophyll and carotenoid concentrations were calculated according to the formula:

$$\text{chlorophyll} = \frac{0.22\ D\ (20.21A_{645} + 8.02A_{663})}{m}$$

$$\text{carotenoid} = \frac{0.22\ D\ (1000A_{470} - 2372.8A_{645} + 445.19A_{663})}{229\ m}$$

D: dilution ratio; m: sample mass.

Abbreviations

AII	*Agrobacterium*-mediated injection-infiltration
AVI	*Agrobacterium*-mediated vacuum-infiltration
CLBV	*Citrus leaf blotch virus*
CTV	*Citrus tristeza virus*
CP	Coat protein
Dpi	Days post inoculation/infiltration
DI	Disease index
VIGS	Virus-induced gene silencing
AVI	*Agrobacterium*-mediated vacuum-infiltration

GFP Green fluorescent protein
HLB Huanglongbing
LOB1 *Lateral organ boundaries1*
ORFs Open reading frames
PDS Phytoene desaturase
SU Sulfur
UTRs Untranslated regions
Xcc *Xanthomonas citri* ssp. *citri*

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Author contributions
CW, ZM, and ZS designed the experiments. CW and ZM performed the experiments. CW and ZM analyzed the data. CW wrote the manuscript. ZM and ZS revised and polished the manuscript. All authors contributed to the article and approved the submitted version.

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Declarations

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The authors declare that they have no competing interests.

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