

Homologues of *CsLOB1* in citrus function as disease susceptibility genes in citrus canker

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SUMMARY

The lateral organ boundary domain (LBD) genes encode a group of plant-specific proteins that function as transcription factors in the regulation of plant growth and development. *Citrus sinensis* lateral organ boundary 1 (*CsLOB1*) is a member of the LBD family and functions as a disease susceptibility gene in citrus bacterial canker (CBC). Thirty-four LBD members have been identified from the *Citrus sinensis* genome. We assessed the potential for additional members of LBD genes in citrus to function as surrogates for *CsLOB1* in CBC, and compared host gene expression on induction of different LBD genes. Using custom-designed transcription activator-like (TAL) effectors, two members of the same clade as *CsLOB1*, named *CsLOB2* and *CsLOB3*, were found to be capable of functioning similarly to *CsLOB1* in CBC. RNA sequencing and quantitative reverse transcription-polymerase chain reaction analyses revealed a set of cell wall metabolic genes that are associated with *CsLOB1*, *CsLOB2* and *CsLOB3* expression and may represent downstream genes involved in CBC.

Keywords: citrus bacterial canker, lateral organ boundary domain (LBD) genes, susceptibility (S) genes.

INTRODUCTION

The lateral organ boundary domain (LBD) genes encode a group of plant-specific proteins that function as transcription factors in the regulation of plant growth and development (Husbands *et al.*, 2007; Majer and Hochholdinger, 2011). All members contain a domain referred to as the lateral organ boundary (LOB) domain, which includes a cysteine-rich motif (CX2CX6CX3C) that has been shown, in some cases, to mediate DNA binding (Husbands *et al.*, 2007; Majer and Hochholdinger, 2011). The proteins also contain a conserved glycine residue and a coiled-coil leucine zipper-like motif (LX6LX3LX6L). *Citrus sinensis* lateral organ boundary 1 (*CsLOB1*) is a member of the

LBD gene family and functions as a disease susceptibility (S) gene in citrus bacterial canker (CBC), a disease that afflicts most citrus varieties worldwide (Hu *et al.*, 2014). The typical symptoms are erumpent water-soaked lesions with yellow haloes on leaves, stems and fruits. Defoliation and premature fruit drop may occur if disease is severe (Gottwald *et al.*, 2002). The causal pathogens are various species of the genus *Xanthomonas*. Five bacterial strain groups (group A, group A*, group Aw, group B and group C) have been identified. Group A, A* and Aw are strains of the species of *Xanthomonas citri* ssp. *citri* (Xcc), which originated from Asia, whereas groups B and C are strains of the species *X. fuscans* ssp. *aurantifolii* (Xau) (Al-Saadi *et al.*, 2007; Sun *et al.*, 2004; Verniere *et al.*, 1998). Pustule formation, regardless of the inciting strain, requires *CsLOB1* expression, which is also associated with increases in bacterial populations at the site of infection (Hu *et al.*, 2014; Pereira *et al.*, 2014).

How *CsLOB1* expression facilitates pustule formation and whether *CsLOB1*-related genes can function in place of *CsLOB1* are unknown. Forty-three LBD genes have been identified in *Arabidopsis* (Shuai *et al.*, 2002). The founding member of the LBD family in *Arabidopsis*, *AtLOB*, is expressed at the base of lateral organs and is involved in leaf development. *AtLOB* functions as a transcription activator and interacts with members of the basic helix–loop–helix (bHLH) family of transcription factors (Husbands *et al.*, 2007; Shuai *et al.*, 2002). Other LBD proteins have been demonstrated to regulate anthocyanin and nitrogen metabolism and to respond to phytohormones and environmental stimuli during the process of organ boundary formation (Gendron *et al.*, 2012; Majer and Hochholdinger, 2011). An LBD gene from apple (*Malus domestica* B.), *MdLBD11*, has a similar function to the *Arabidopsis* gene *AtAS2/AtLBD12* in terms of its role in leaf development and flowering (Wang *et al.*, 2013). The expression of the *Arabidopsis* gene *LBD20* is induced on infection by the pathogen *Fusarium oxysporum*, and silencing of the *LBD20* results in increased resistance to the pathogen (Thatcher *et al.*, 2012).

CsLOB1 induction early in the disease process is mediated by one of the six major transcription activator-like (TAL) effectors from strains of Xcc or Xau (Hu *et al.*, 2014; Pereira *et al.*, 2014). PthA was the first member of the TAL effector family demonstrated to function in pathogenicity and shown to be required for pustule formation (Swarup *et al.*, 1992). Additional *pthA* homologous genes have been identified

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from other strains of *Xanthomonas* and include *pthA4*, *pthAw*, *pthA**, *pthB* and *pthC* (Al-Saadi *et al.*, 2007; Jalan *et al.*, 2013; Sun *et al.*, 2004; Verniere *et al.*, 1998). All of the major TAL effectors target *CsLOB1* (Hu *et al.*, 2014; Pereira *et al.*, 2014). TAL effectors are members of the type III secretion (T3S) effectors primarily identified in *Xanthomonas* species and *Ralstonia solanacearum* (De Feyter *et al.*, 1993; Heuer *et al.*, 2007; Hopkins *et al.*, 1992; Mak *et al.*, 2013). TAL effectors, on entry into host cells, move to the cell nuclei and bind to the specific promoter regions, designated here as effector-binding elements (EBEs), of host genes. The expression of a variety of genes that are TAL effector-dependent leads to either enhanced susceptibility or host resistance, or, in some cases, no clear phenotypic effect on the plant (Boch *et al.*, 2014; Zhang *et al.*, 2015). The cognate EBE is determined by the TAL effector central repetitive region. The amino acid sequence of the repeats is largely conserved with the exception of residues 12 and 13, which are polymorphic and referred to as repeat-variable diresidues (RVDs) (Boch *et al.*, 2009, 2014; Moscou and Bogdanove, 2009).

Artificially designed TAL effector (dTALe) genes can be produced by replacing the repetitive region with coding segments that target new binding sites of interest (Li *et al.*, 2013; Morbitzer *et al.*, 2010; Streubel *et al.*, 2013). The targeting of novel promoter elements by dTALes has been proven to be useful for the discrimination between *S* genes and phenotypically silent or off-target genes that also contain native EBEs (Cernadas *et al.*, 2014; Cohn *et al.*, 2014; Hu *et al.*, 2014). For example, PthA4 and PthAw both induce *CsLOB1* and a second gene *CsSWEET1* (Hu *et al.*, 2014). The construction of dTALes that targeted either gene revealed that only the induction of *CsLOB1* contributed to pustule formation and enhanced population levels of the pathogen (Hu *et al.*, 2014; Pereira *et al.*, 2014). Furthermore, dTALes facilitate the identification of *S* gene surrogates, especially for the paralogues of the identified *S* genes. TAL effectors of field strains of *X. oryzae* pv. *oryzae* are known to target any one of three SWEET genes (*OsSWEET11*, *OsSWEET13*, *OsSWEET14*) encoding closely related members of clade III of sucrose transporters in rice (Antony *et al.*, 2010; Yang *et al.*, 2006; Zhou *et al.*, 2015). The use of dTALes allowed the targeting of an additional 19 *OsSWEET* genes. However, only two additional members (*OsSWEET12* and *OsSWEET15*) from clade III could induce the typical disease symptom of water-soaked lesions, revealing that only members of clade III can function as *S* genes (Streubel *et al.*, 2013). Here, we assessed the potential for additional members of the *LBD* genes in citrus to function as surrogates for *CsLOB1* in CBC, and compared host gene expression on induction of different *LBD* genes.

RESULTS

Phylogeny of citrus *LBD* gene family

Thirty-four members in the *C. sinensis* *LBD* family were identified in the Plant Transcription Factor Database (<http://planttfdb.cbi.pku.edu.cn/>

family.php?sp=Csi&fam=LBD) (File S1, see Supporting Information). The three citrus genes most closely related to *CsLOB1* based on the *C. sinensis* proteome are orange1.1g047491m, orange1.1g045080m and orange1.1g036782m according to their BLAST scores. They share 71.0%, 67.9% and 71.9% identities to *CsLOB1*, respectively. None has been characterized functionally, with the exception of the function of *CsLOB1* (orange1.1g026556m) in CBC (Hu *et al.*, 2014). The constructed phylogenetic tree was divided into nine clades. The clade with *CsLOB1*, designated as clade I, harbours four additional members—orange1.1g047491m, orange1.1g045080m, orange1.1g047530m and orange1.1g047804m (Fig. 1). Two of the four clade I *LBD* proteins (orange1.1g045080m and orange1.1g047491m) and one (orange1.1g036782m) from clade III were chosen for dTALe targeting (Fig. 1, red arrows). Hereafter, the three *LBD* genes (orange1.1g045080m, orange1.1g047491m and orange1.1g036782m) are designated as *CsLOB2*, *CsLOB3* and *CsLOB4*, respectively.

Pustule symptoms are restored by dTALes targeting *CsLOB2* and *CsLOB3*, but not *CsLOB4*

A set of dTALes was designed to target the promoters of *CsLOB2*, *CsLOB3* and *CsLOB4*, which were designated as d*CsLOB2*, d*CsLOB2L*, d*CsLOB3* and d*CsLOB4*, respectively (Fig. S1, see Supporting Information; Table 1). The functionality of each dTALe was tested by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of mRNA from grapefruit leaves after inoculation with a *pthA4* mutant strain of Xcc-306 (*Xcc306ΔpthA4*) and the same strain complemented by introduction of the individual dTALe genes (d*CsLOB2*, d*CsLOB2L*, d*CsLOB3* and d*CsLOB4*). Compared with *Xcc306ΔpthA4*, the complemented strains induced higher fold changes for the respective gene targets (Figs 2a and S6b, see Supporting Information).

The contribution of the respective strains harbouring dTALes for the respective targeted *LBD* genes to disease symptom development was tested on grapefruit leaves. At 15 days post-inoculation (dpi), the inoculation sites treated with bacterial suspensions of *Xcc306WT*, *Xcc306ΔpthA4::dCsLOB2*, *Xcc306ΔpthA4::dCsLOB2L* or *Xcc306ΔpthA4::dCsLOB3* showed pustule formation (Fig. 3a,b, panels 1–3, and Fig. S6a, panel 1, respectively), whereas the sites treated with bacterial suspensions of either *Xcc306ΔpthA4* or *Xcc306ΔpthA4::dCsLOB4* only showed water-soaked lesions (Fig. 3b, panels 4 and 5). To determine whether induction of an alternative *LBD* member is also associated with an increase in the bacterial population on citrus leaves, bacterial population assays were conducted with strains of *Xcc306WT* (with *pthA4*), *306ΔpthA4* and *Xcc306ΔpthA4::dCsLOB2*. Indeed, *Xcc306ΔpthA4::dCsLOB2* achieved higher populations than *306ΔpthA4* and was comparable with strain *Xcc306WT* after 3 days on host plants (Fig. 4).

To exclude the possibility that pustule formation or pathogen growth conferred by *CsLOB2* or *CsLOB3* was caused by the

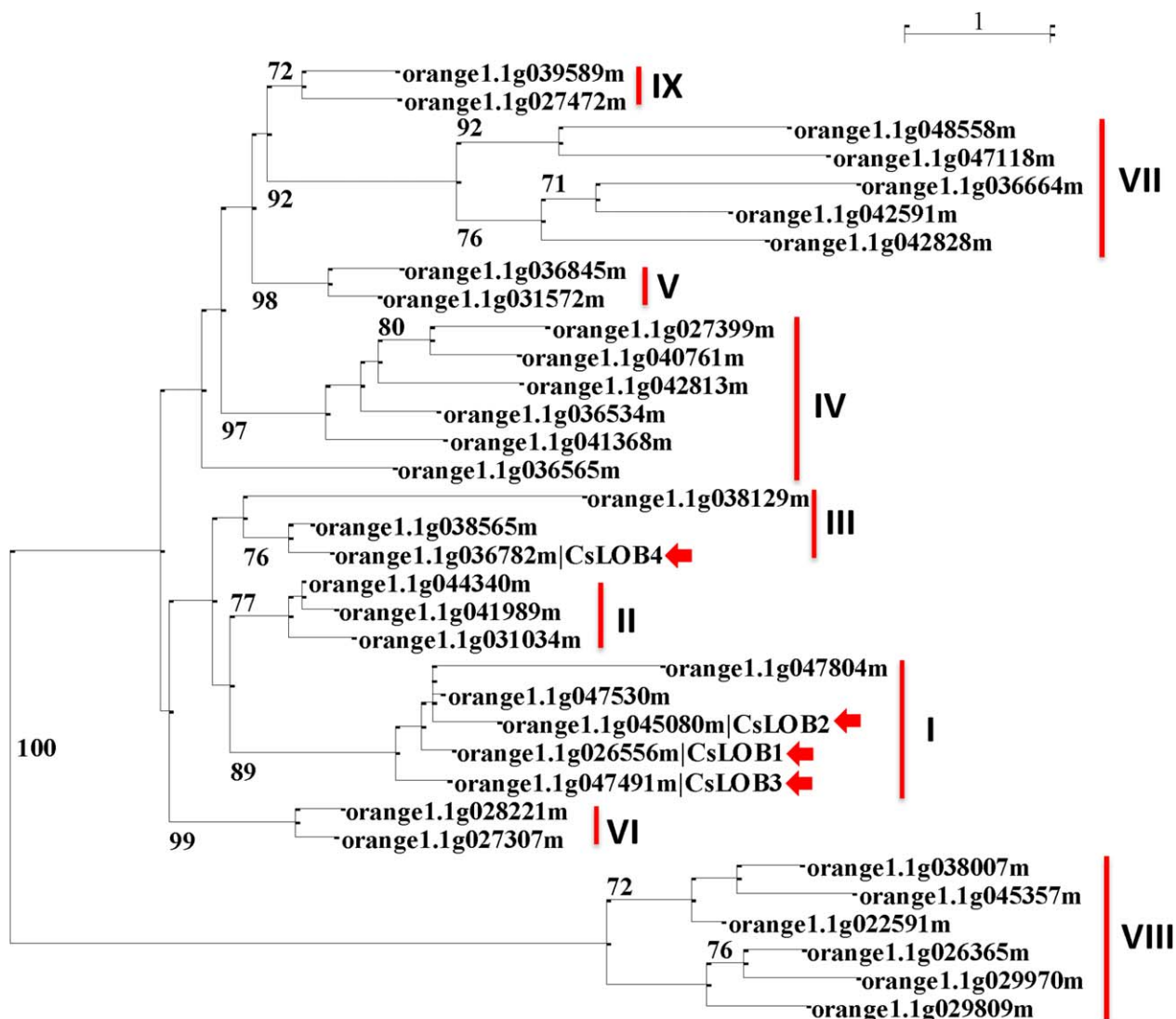


Fig. 1 Phylogenetic tree of *Citrus sinensis* lateral organ boundary domain (LOB) genes. Values with a cutoff of 70 for the bootstrap support (500 replicates) are shown on the shoulders of the branches. The scale bar indicates substitutions/site, and vertical lines are used to represent different clades of *C. sinensis* LBD proteins.

coincidental expression of *CsLOB1*, the induction of *CsLOB1* in the presence of *dCsLOB2* or *dCsLOB3* was also assayed. No significant induction of *CsLOB1* by *Xcc306ΔpthA4::dCsLOB2* or *Xcc306ΔpthA4::dCsLOB3* relative to *Xcc306ΔpthA4* was observed (Fig. 2b).

S gene expression does not contribute to enhanced virulence of a non-host pathogen

Xanthomonas campestris pv. *vesicatoria* (Xcv) Xcv85-10, a pathogen of pepper, was used as a non-host strain to determine whether a TAL effector gene could alter the virulence of Xcv85-10 on a non-host. Xcv85-10 normally induces light water soaking on citrus (Fig. 5a). *CsLOB2* was highly induced by Xcv85-10::*dCsLOB2* when inoculated on grapefruit leaves compared

with the control Xcv85-10::pHM1 (empty vector) as determined by qRT-PCR (Fig. S2, see Supporting Information). Pustule-like symptoms were observed at the inoculation sites with Xcv85-10::*dCsLOB2* (Fig. 5a, panels 4 and 6). However, Xcv85-10, which grows to relatively low population levels compared with Xcc306WT, did not grow to higher populations in the presence vs. absence of *dCsLOB2* (Fig. 5b).

Comparison of genes induced in association with *CsLOB1*, *CsLOB2*, *CsLOB3* or *CsLOB4* expression

CsLOB1, *CsLOB2* and *CsLOB3* might induce CBC development by the regulation of common downstream genes and pathways. RNA sequencing (RNA-Seq) was performed to investigate the

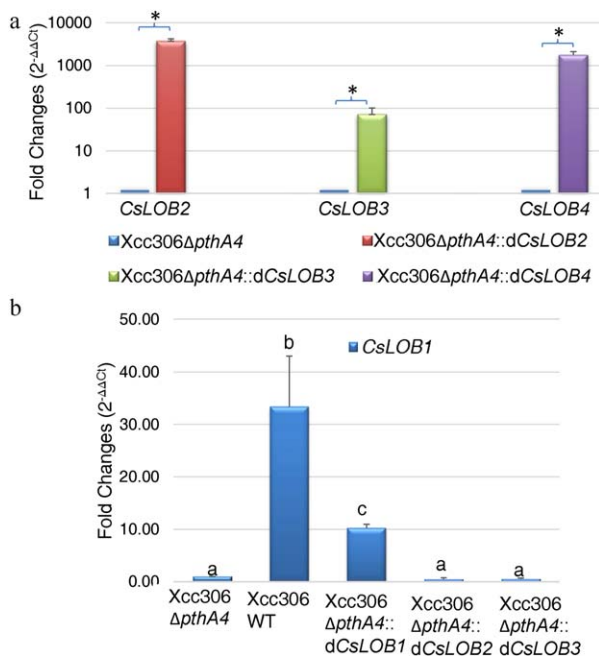


Fig. 2 The targeted *lateral organ boundary domain* (*LBD*) genes are induced by the respective designed transcription activator-like effectors (dTALes). (a) Validation of targeted gene induction by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Asterisks denote significant differences in the fold changes between the treatments relative to the negative control Xcc306ΔpthA4 at $P < 0.05$ using a t -test. (b) *CsLOB1* is not induced by the dTALes targeting *CsLOB2* or *CsLOB3*. Different letters indicate significant differences in fold changes among the treatments at $P < 0.05$ using analysis of variance (ANOVA). qRT-PCR was conducted on mRNA extracted from grapefruit leaves inoculated with the individual *Xanthomonas citri* ssp. *citri* (Xcc) strains as indicated using *LBD* gene-specific primers at 5 days post-inoculation (dpi). Expression values were normalized to the housekeeping gene orange1.1g001725m. Error bars represent the standard deviations for three independent experimental replicates. Samples were taken at 5 dpi.

transcriptional profile of infected tissues with strains of the pathogen that induce any one of the genes *CsLOB1*, *CsLOB2*, *CsLOB3* or *CsLOB4*. Grapefruit leaves were separately inoculated with Xcc306ΔpthA4 (negative control), Xcc306WT, which harbours *pthA4* and induces *CsLOB1*, Xcc306ΔpthA4::dCsLOB2, Xcc306ΔpthA4::dCsLOB3 and Xcc306ΔpthA4::dCsLOB4 (data uploaded at Sequence Read Archive with accession number SRP066665). Comparisons of gene expression were conducted between Xcc306ΔpthA4 and each of Xcc306WT, Xcc306ΔpthA4::dCsLOB2, Xcc306ΔpthA4::dCsLOB3 and Xcc306ΔpthA4::dCsLOB4, resulting in 3748, 3892, 4749 and 466 significantly differentially expressed genes (DEGs), respectively, with a control of 5% false discovery rate (FDR) for each comparison. Furthermore, 1797 common DEGs were identified, with 737 up-regulated and 977 down-regulated, in all three comparisons: Xcc306WT vs. Xcc306ΔpthA4, Xcc306ΔpthA4::dCsLOB2 vs. Xcc306ΔpthA4 and Xcc306ΔpthA4::dCsLOB3 vs. Xcc306ΔpthA4. Among the 1797 genes, 163 genes were significantly up- or down-regulated in all four

comparisons. Thus, 1634 genes were associated with the expression of *CsLOB1*, *CsLOB2* and *CsLOB3*, but not with the expression of *CsLOB4* (Fig. 6).

Mercator data analysis was used to predict the pathways associated with the expression of each one of the four *LBD* genes. For the up-regulated genes, a threshold of fold change greater than two was applied as a filter to the 737 genes associated with the expression of *CsLOB1*, *CsLOB2* and *CsLOB3*, which yielded 466 up-regulated common genes. The gene annotations were clustered into categories of cell, cell wall, protein, RNA and signalling (Fig. S3a, File S2, see Supporting Information). Cell and cell wall were the two categories apportioned to most of the genes. Genes in the cell category were mostly classified into cell organization, cell division and cell cycle. The cell wall genes were mostly apportioned into cell wall degradation and cell wall modification (File S2).

A cutoff of fold change greater than two was also utilized to filter the 977 significantly down-regulated genes associated with the expression of *CsLOB1*, *CsLOB2* and *CsLOB3*, and 564 genes were identified. Mercator analysis of these 564 transcripts showed that most of the transcripts were classified into categories of signalling, stress, protein, hormone metabolism and secondary metabolism (Fig. S3b, File S3, see Supporting Information). Signalling and stress were the groups with the largest number of genes. Most of the signalling category genes were receptor kinase signalling and calcium signalling genes, and genes in the stress category were mostly grouped into biotic stress (File S3).

Expression assays were conducted using qRT-PCR to validate the RNA-Seq data for a selected set of genes. Most of the induced cell wall-related genes were categorized into cell wall degradation and cell wall modification according to the Mercator analysis results (Table S1, see Supporting Information). Eight representative genes for cell wall degradation and modification with high fold changes were selected for validation by qRT-PCR analysis. Five were cell wall degradation genes, including an endo- β -1,4-glucanase gene (orange1.1g009690m), a glycosyl hydrolase family 5 gene (orange1.1g014426m), one polygalacturonase gene (orange1.1g043061m) and two pectate lyase genes (orange1.1g015623m and orange1.1g011814m). Three were cell wall modification genes, including two β -expansin 3 genes (orange1.1g023962m and orange1.1g024303m) and an α -expansin 9 gene (orange1.1g024916m). RNA samples were prepared from independent inoculations of grapefruit leaves treated with Xcc306ΔpthA4, Xcc306WT, Xcc306ΔpthA4::dCsLOB2, Xcc306ΔpthA4::dCsLOB3 or Xcc306ΔpthA4::dCsLOB4. The selected cell wall-related genes were up-regulated in response to inoculations by Xcc306WT, Xcc306ΔpthA4::dCsLOB2 or Xcc306ΔpthA4::dCsLOB3 relative to Xcc306ΔpthA4; five were also up-regulated by Xcc306ΔpthA4::dCsLOB4 relative to Xcc306ΔpthA4, but the remaining three were not (Fig. 7). The results indicated that the expression of *CsLOB1*, *CsLOB2* and *CsLOB3* was associated with cell wall-related gene expression. The eight selected cell wall-related genes were all induced by

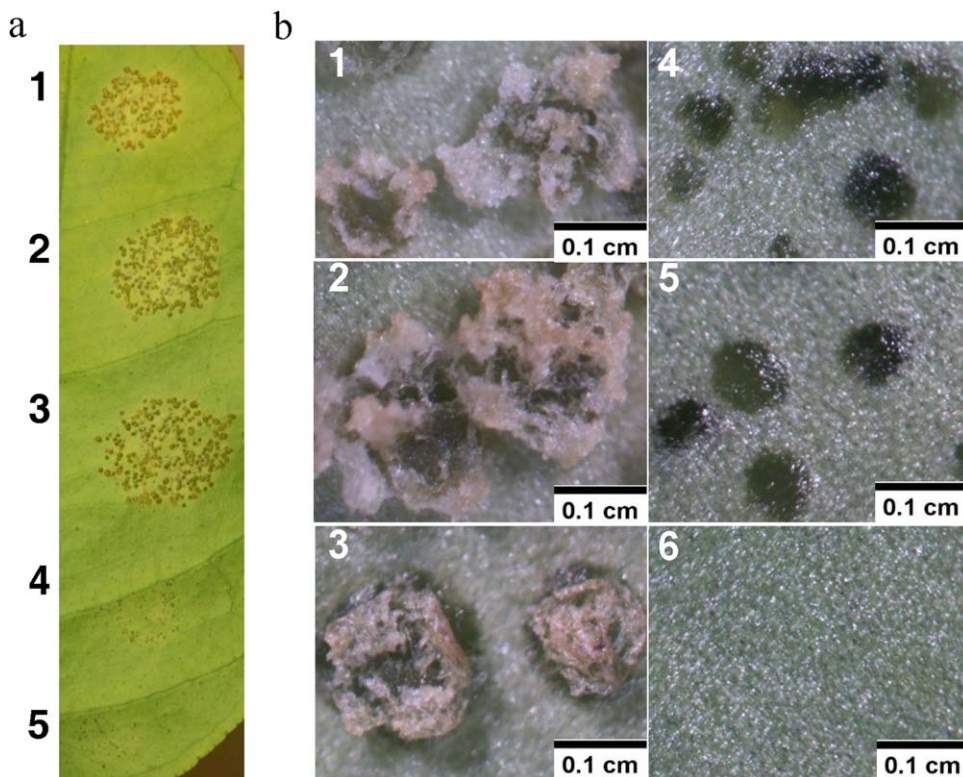


Fig. 3 Disease symptoms induced by designed transcription activator-like effectors (dTALEs) targeting the three *CsLOB1*-related genes. (a) Light photography at 1 \times ; (b) 6.4 \times magnification. Panels and inoculation sites: 1, *Xcc306WT*; 2, *Xcc306 Δ pthA4::dCsLOB2*; 3, *Xcc306 Δ pthA4::dCsLOB3*; 4, *Xcc306 Δ pthA4::dCsLOB4*; 5, *Xcc306 Δ pthA4*; 6, healthy leaf. Photographs were taken at 15 days post-inoculation (dpi) of various strains as shown in the figures. Inocula were 10^3 times dilutions of bacterial suspensions with an optical density at 600 nm (OD_{600}) of 0.5.

Xcc306WT, *Xcc306 Δ pthA4::dCsLOB2* or *Xcc306 Δ pthA4::dCsLOB3* relative to *Xcc306 Δ pthA4* at significance levels of $P < 0.05$. Although the expression levels of the eight genes increased in association with

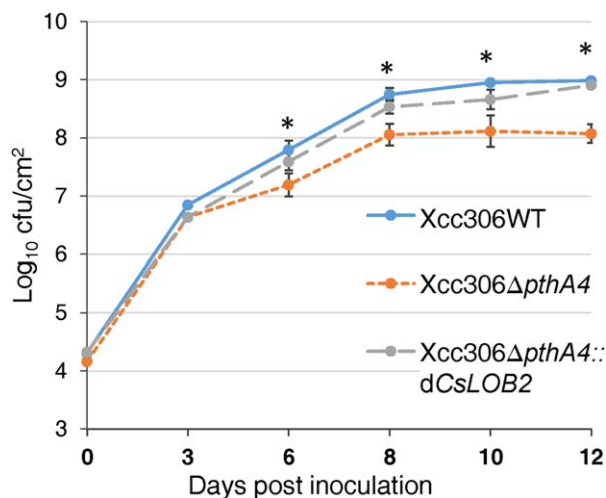


Fig. 4 *dCsLOB2* promoted an increase in the bacterial population in host plants. Bacterial populations were measured for three strains (*Xcc306 Δ pthA4*, *Xcc306WT* and *Xcc306 Δ pthA4::dCsLOB2*) on grapefruit. The *x*-axis shows the days post-inoculation. Asterisks indicate significant difference ($P < 0.01$) of two comparisons: *Xcc306WT* and *Xcc306 Δ pthA4::dCsLOB2* vs. *Xcc306 Δ pthA4*. Statistical significance of the analysis was tested using *t*-test. cfu, colony-forming unit.

CsLOB4 expression, the levels were, at a maximum, elevated to one-fifth of the level associated with *CsLOB3* expression (Fig. 7). Three of the selected cell wall-related genes, which were exclusively up-regulated by expression of *CsLOB1*, *CsLOB2* and *CsLOB3*, included one polygalacturonase gene (orange1.1g043061m), one pectate lyase gene (orange1.1g011814m) and one β -expansin gene (orange1.1g024303m) (Fig. 7).

To exclude the possibility that the induction of genes by *Xcc306 Δ pthA4::dCsLOB4* relative to *Xcc306 Δ pthA4* might be a result of direct induction by *dCsLOB4*, a second dTALE, *dCsLOB4-2*, was constructed, targeting a different EBE in the promoter region of *CsLOB4* (Fig. S1). After introduction of *dCsLOB4-2* into *Xcc306 Δ pthA4*, *CsLOB4* was efficiently induced, and no pustule formation was observed (Fig. S5a,b). Nine genes whose log₂ fold changes (*Xcc306 Δ pthA4::dCsLOB4* vs. *Xcc306 Δ pthA4*) were greater than two were selected to perform the qRT-PCR validation using both *Xcc306 Δ pthA4::dCsLOB4* and *Xcc306 Δ pthA4::dCsLOB4-2*. qRT-PCR results revealed that four of the nine genes were significantly induced by both *Xcc306 Δ pthA4::dCsLOB4* and *Xcc306 Δ pthA4::dCsLOB4-2* relative to *Xcc306 Δ pthA4* (Fig. 8).

DISCUSSION

The results indicate that more than one member of the LBD genes could potentially serve as an *S* gene in CBC. Two LBD family members, *CsLOB2* and *CsLOB3*, were capable of directing pustule

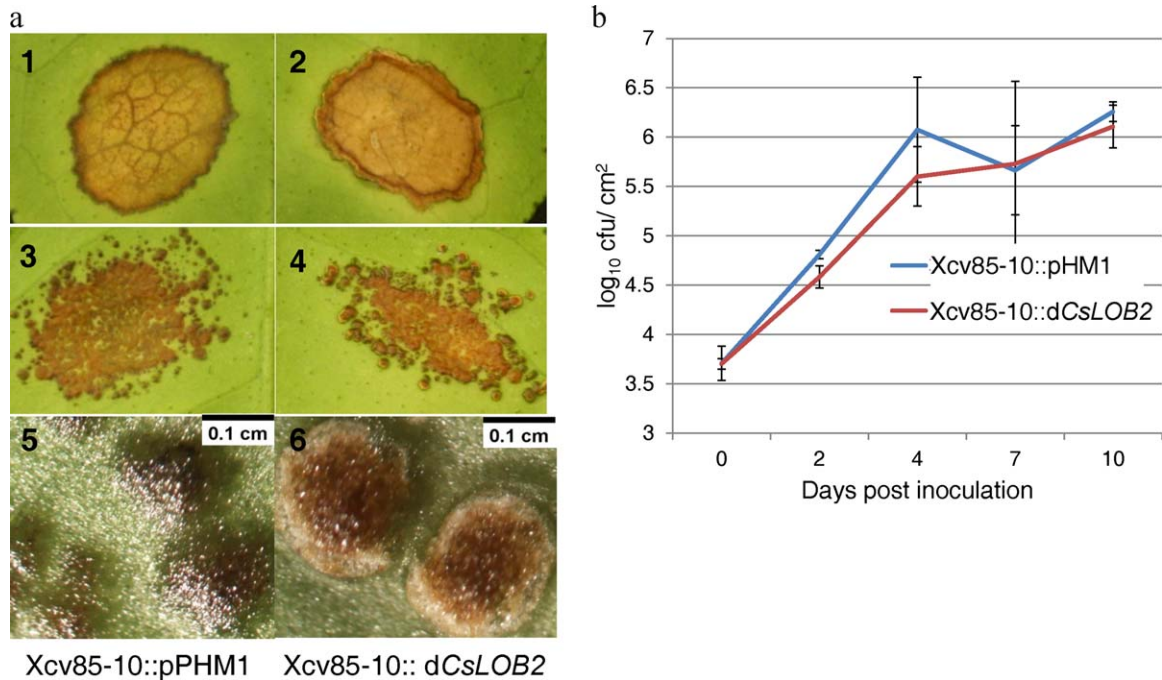


Fig. 5 *S* gene expression does not contribute to enhanced virulence of a non-host pathogen. (a) Pustule-like symptoms were observed when *DCsLOB2* was transformed into *Xanthomonas euvesicatoria* Xcv85-10. Photographs were taken at 30 days post-inoculation (dpi) of grapefruit leaves with Xcv85-10::pPHM1 or Xcv85-10::dCsLOB2 at 1× (1–4) or 6.4× (5, 6) magnification. Inoculum with an optical density (OD₆₀₀) of 0.5 (1,2) or the same inoculum diluted 10³ times (3–6). (b) Bacterial population assay of the two strains Xcv85-10::pPHM1 and Xcv85-10::dCsLOB2 on grapefruit. *t*-test ($P < 0.05$) showed no significant difference between the two treatments. cfu, colony-forming unit.

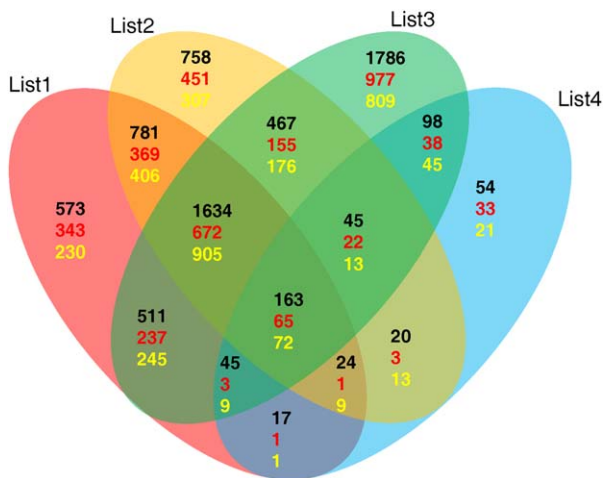


Fig. 6 RNA-sequencing (RNA-Seq) results of the gene expression profile induced by *CsLOB1*, *CsLOB2*, *CsLOB3* or *CsLOB4*. Venn diagrams show the number of genes that were significantly differentially expressed in each comparison (List1, Xcc306WT vs. 306Δ*pthA4*; List2, 306Δ*pthA4*::d*CsLOB2* vs. 306Δ*pthA4*; List3, 306Δ*pthA4*::d*CsLOB3* vs. 306Δ*pthA4*; List4, 306Δ*pthA4*::d*CsLOB4* vs. 306Δ*pthA4*). The comparisons are represented by blue, yellow, green or red ovals. Genes that show significant differences in more than one comparison are plotted in the overlapping areas. The black numbers denote the total significantly regulated genes at a false discovery rate (FDR) < 0.05, the red numbers denote the up-regulated genes and the yellow numbers denote the down-regulated genes.

formation when strains of Xcc containing LBD-specific dTAL genes were inoculated on citrus leaves. TAL effector-mediated induction of *CsLOB4* failed to support pustule formation, indicating that at least one member was incapable of supporting pustule formation. *CsLOB2* and *CsLOB3* both encode members of the same clade as *CsLOB1*, whereas *CsLOB4* lies in a close, but distinct, clade of LBD family members. At the same time, it is premature to conclude that pustule formation is limited to clade I members, as only one member outside of clade I was tested. The induction of *CsLOB1*, *CsLOB2* and *CsLOB3* by the respective TAL effectors facilitated symptom development and, at least in the case of *CsLOB1* and *CsLOB2*, enhanced pathogen population levels in leaves. The results may reflect a common biochemical function of the genes within a specific clade, as observed for the SWEET genes in bacterial blight of rice (Streubel *et al.*, 2013). In contrast with bacterial blight of rice, where three SWEET genes are targeted by field strains, only one member of LBD clade I has been shown to be targeted for CBC in the field (Antony *et al.*, 2010; Hu *et al.*, 2014; Pereira *et al.*, 2014; Yang *et al.*, 2006; Zhou *et al.*, 2015).

The lack of alternative *S* gene targeting in the field might be attributed to the breeding histories of cultivated rice and citrus varieties. Genetic breeding for rice can be traced back 10 000 years (Asano *et al.*, 2011). The repeated deployment of genes for resistance in rice to bacterial blight for specific TAL effector genes in

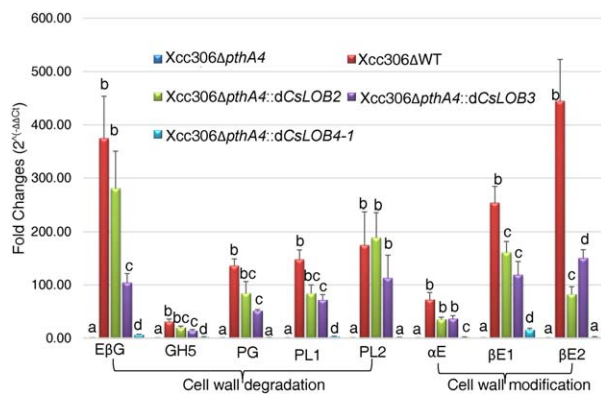


Fig. 7 Quantitative reverse transcription-polymerase chain reaction (RT-PCR) validation of cell wall-related genes induced by the expression of *CsLOB1*, *CsLOB2*, *CsLOB3* and *CsLOB4*. RNA samples were grapefruit leaves treated with various *Xanthomonas citri* ssp. *citri* (Xcc) strains as indicated in the figure, and were taken at 5 days post-inoculation (dpi). Inoculum had an optical density at 600 nm (OD_{600}) of 0.5. Expression values were normalized to the housekeeping gene orange1.1g001725m. Error bars represent the standard error for three independent experimental replicates. Different letters indicate significant differences among the various treatments for each test gene at the significance level of $P < 0.05$ according to analysis of variance (ANOVA). Different genes are represented by abbreviations of the annotations of various transcripts. Gene IDs are from Phytozome. EβG, endo β-1,4-glucanase (orange1.1g009690m); GH5, glycosyl hydrolase family 5 (orange1.1g014426m); PG, polygalacturonase (orange1.1g043061m); PL1, pectate lyase1 (orange1.1g015623m); PL2, pectate lyase2 (orange1.1g011814m); αE, α-expansin (orange1.1g024916m); βE1, β-expansin1 (orange1.1g023962m); βE2, β-expansin2 (orange1.1g024303m).

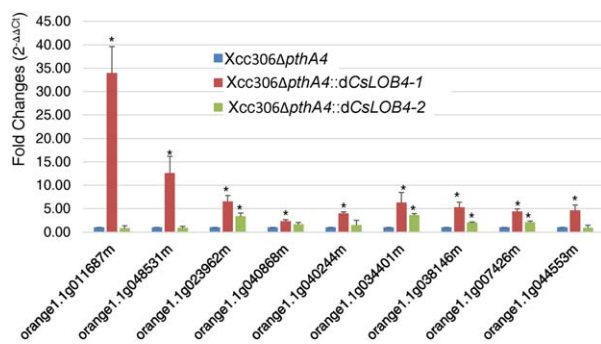


Fig. 8 Quantitative reverse transcription-polymerase chain reaction (RT-PCR) validation of genes up-regulated by the expression of *CsLOB4*, which was induced by either *dCsLOB4* or *dCsLOB4-2*. RNA samples were grapefruit leaves treated with various *Xanthomonas citri* ssp. *citri* (Xcc) strains as indicated in the figure, and were taken at 5 days post-inoculation (dpi). Inoculum had an optical density at 600 nm (OD_{600}) of 0.5. Expression values were normalized to the housekeeping gene orange1.1g001725m. Different genes are represented by the transcript ID from Phytozome. Error bars represent the standard error for three independent experimental replicates. Single asterisks indicate a significant difference relative to 306ΔpthA4 at the significance level of $P < 0.05$ according to *t*-test.

X. oryzae pv. *oryzae*, and the long breeding history, undoubtedly produced allelic *S* gene targets and selective pressure for new TAL effector specificities (Garris *et al.*, 2005; Hutin *et al.*, 2015; Shinada *et al.*, 2014). The documented breeding history of citrus is for a much shorter time, perhaps 200 years, and, as a tree species, breeding cycles are considerably longer (Khan, 2007). Therefore, the genetic complexity of the bacterial–plant interactions may be much lower in citrus than in rice, putting less selective pressure on the pathogen population and TAL effector evolution. An alternative model is that the disease itself is rather young, resulting from the recent horizontal transfer of a TAL effector gene. A variety of *Xanthomonas* strains do not induce a strong non-host hypersensitive reaction in citrus and cause bacterial spot-like symptoms (Gent *et al.*, 2005). CBC strains, in addition to a single *S* gene target, have relatively fewer copies of TAL effector genes within a single strain in comparison with *X. oryzae* pv. *oryzae*, which can have as many as 19 genes in a single strain, and the genes are often plasmid-borne, providing further evidence of recent acquisition (Al-Saadi *et al.*, 2007; Salzberg *et al.*, 2008; da Silva *et al.*, 2002).

CBC pustule formation has been observed previously on transfer of *pthA* from *X. citri* pv. *citri* to *Xanthomonas* pathovars, although the growth within a new host was not assessed (Swarup *et al.*, 1991). Here, the *dCsLOB2* gene was transferred into *X. euvesicatoria* Xcv85-10, which is a pathogen of pepper and causes slight water-soaking symptoms in Duncan grapefruit. Xcv85-10 with *dCsLOB2* also induced pustules in grapefruit leaves. However, no differences were observed in pathogen leaf populations compared with populations of Xcv85-10 without *dCsLOB2*, indicating that the presence of pustule-associated TAL effector genes does not, in itself, override other requirements for host adaptation. From a practical standpoint, the results also indicate that the CBC causal strains are broadly at risk, at least in the short term, of recessive resistance strategies involving engineering polymorphisms in the *CsLOB1* promoter, similar to changes in the EBEs of rice *S* gene promoters (Antony *et al.*, 2010; Li *et al.*, 2012; Yang *et al.*, 2006; Zhou *et al.*, 2015)

Little is known about the biological functions of LBD members in citrus. The constructed phylogenetic tree (Fig. S4a, see Supporting Information) shows that *CsLOB1*, *CsLOB2* and *CsLOB3* are most closely related to *AtLBD1* and *AtLBD11*, whereas *CsLOB4* is more closely related to *AtLBD12*. The protein sequence alignment reveals the consensus LOB domain that contains the three conserved motifs: a cysteine-rich domain CX2CX6CX3C, a conserved glycine residue and a coiled-coil leucine zipper-like motif LX6LX3LX6L (Fig. S4b). *AtLBD12* and *CsLOB4* have much shorter N-terminals, which might account for the dysfunction of *CsLOB4* as an *S* gene in CBC. However, reports on the function of *AtLBD1* or *AtLBD11* are few (Matsui *et al.*, 2008). *AtLBD12* mutants have been reported to show reduced leaf size, apical dominance and epinastic leaves, and the mutant plants show increased sterility

(Nakazawa *et al.*, 2003). Therefore, the prediction of the biological functions of *CsLOB1*, *CsLOB2* and *CsLOB3* is premature based on homology.

Citrus LBD genes potentially function in the regulation of downstream pathways for normal plant development. RNA-Seq analysis of the expression profiles associated with *CsLOB1*, *CsLOB2* and *CsLOB3* exposed a common set of downstream genes; genes in categories of cell organization, cell division, cell cycle, cell wall degradation and cell wall modification were significantly up-regulated. However, genes in classes of receptor kinase signalling, calcium signalling and biotic stress were mainly down-regulated, which indicates that *CsLOB1*, *CsLOB2* and *CsLOB3* might regulate this set of downstream genes which ultimately trigger pustule development.

The reason why the induction of *CsLOB4* could not restore the pustule formation ability of the *pthA4* mutant strain is not clear. First, the possibility that *CsLOB4* protein is dysfunctional cannot be excluded. Four hundred and sixty-six genes were significantly induced by *dCsLOB4*. Nine genes showed a log₂ fold change (*Xcc306ΔpthA4::dCsLOB4* vs. *Xcc306ΔpthA4*) greater than two, some of which were also associated with the expression of the other three LBD genes (Table 2). Furthermore, four genes were induced by both *Xcc306ΔpthA4::dCsLOB4* and *Xcc306ΔpthA4::dCsLOB4-2* relative to *Xcc306ΔpthA4*, as revealed by qRT-PCR analysis, which reduces the likelihood that the induced genes might be off-targets of dTAles and indicates that *CsLOB4* is not dysfunctional. However, the low efficiency of *CsLOB4* as a transcription factor might be the reason why the expression of *CsLOB4* could not induce pustule formation. Expression of *CsLOB4* was shown to be associated with a much smaller set of downstream genes compared with those related to the expression of the other three pustule-inducible LBD members. Although there are 163 genes that are commonly associated with the expression of all four LBD genes, for most of the genes, the absolute log₂ fold changes in the comparison of *Xcc306ΔpthA4::dCsLOB4* vs. *Xcc306ΔpthA4* are lower than those in the other three comparisons (File S4, see Supporting Information).

The results revealed that a subset of cell wall-related genes, especially those involving cell wall degradation and cell wall modification, were induced when the pustule-associated LBD genes were present. Induction of *CsLOB4*, one member incapable of forming pustules, was associated with much lower levels of expression or no induction of the cell wall-related genes, which indicates that these cell wall-related genes may be functionally important for pustule formation and symptom development. The plant cell wall acts as a physical barrier in interactions with plant pathogens, and pathogens employ various strategies to manipulate their host plant cell wall metabolism to favour infection (Bellincampi *et al.*, 2014). In tomato, several cell wall-related genes, including *Cel1* and *Cel2* (endo-β-1,4-glucanase), *LePG*

(polygalacturonase) and *LeExp1* (expansin), were up-regulated during infection by *Botrytis cinerea*. Silencing of the genes attenuated the susceptibility of tomato fruits to *B. cinerea* (Cantu *et al.*, 2009; Flors *et al.*, 2007). The expression of *CsLOB1*, *CsLOB2* and *CsLOB3* may reflect the role played by a set of cell wall metabolic genes as susceptibility factors. Targeting of individual downstream citrus genes may allow the identification of specific critical genes using the dTAle strategy.

The clade I LBD genes of citrus join a limited club of TAL effector-dependent S genes that have been shown to contribute major phenotypic features to the respective disease processes. Three other examples have been characterized. The only one for bacterial spot of pepper is *upa20*, encoding a bHLH family transcription factor, the S gene targeted by TAL effector AvrBs3 from *X. campestris* pv. *vesicatoria*. UPA20 has been revealed to play possible roles in cell enlargement in pepper bacterial leaf spot disease (Kay *et al.*, 2007). *OsSULTR3;6*, a putative sulfate transporter gene, is targeted by Tal2g of *X. oryzae* pv. *oryzicola* in rice bacterial leaf streak disease, where the gene facilitates lesion expansion and bacterial exudation (Cernadas *et al.*, 2014). The largest group is the SWEET genes of rice and cassava (Antony *et al.*, 2010; Cohn *et al.*, 2014; Streubel *et al.*, 2013; Yang *et al.*, 2006; Zhou *et al.*, 2015). These SWEET proteins have been demonstrated to function in the transport of sucrose or glucose during pathogenesis (Chen *et al.*, 2010, 2012; Zhou *et al.*, 2015). The range of genes that have been identified to date indicates that a variety of host genes can serve as S genes. At the same time, little is known about the function of TAL effector-dependent S genes from a physiological perspective. The characterization of additional pathosystems that involve TAL effector-mediated virulence may ultimately reveal common pathways for the enhancement of host susceptibility.

EXPERIMENTAL PROCEDURES

Plant material, bacterial strains and plasmids

Grapefruit trees were grown at 28 °C, 80% humidity, at a setting of 16 h of daylight and 8 h of darkness. All *Xanthomonas* strains were incubated at 28 °C, and *Escherichia coli* strains were cultured at 37 °C. All the bacterial strains and plasmids are shown in Table 1. The *E. coli* competent cells used were Stellar™ Competent Cells from Clontech Mountain View, CA, USA or NEB 5-alpha Competent *E. coli* (High Efficiency) from New England Biology (Ipswich, MA, USA). The *Xanthomonas* competent cells were prepared by washing the cultured bacteria with 10% glycerol three times, and transformed using the electroporation method (Alexandre *et al.*, 2005).

Bacterium inoculation

Xanthomonas strains were streaked on tryptone sucrose agar (TSA) medium incubated at 28 °C for 48 h. Single colonies were picked, spread on new TSA plates and incubated at 28 °C for 2 days. The bacteria were

Table 1 Strains and plasmids used in this study.

| Strain or plasmid | Feature | Source |
|--|---|---|
| <i>Xanthomonas citri</i> ssp. <i>citri</i> Xcc306WT Xcc306 Δ <i>pthA4</i> | Xcc306 Group A, wild-type, Rif <i>pthA4</i> deletion mutant | DPI* Jeffery Johnes's laboratory (Gainesville, FL, USA) |
| Xcc306 Δ <i>pthA4</i> ::d <i>CsLOB1</i> | Artificial TALE targeting <i>CsLOB1</i> complement Xcc306 Δ <i>pthA4</i> , Gm | This study |
| Xcc306 Δ <i>pthA4</i> ::d <i>CsLOB2</i> | Artificial TALE targeting <i>CsLOB2</i> complement Xcc306 Δ <i>pthA4</i> , Gm | This study |
| Xcc306 Δ <i>pthA4</i> ::d <i>CsLOB2L</i> | Artificial TALE targeting <i>CsLOB2</i> complement Xcc306 Δ <i>pthA4</i> , Gm | This study |
| Xcc306 Δ <i>pthA4</i> ::d <i>CsLOB3</i> | Artificial TALE targeting <i>CsLOB3</i> complement Xcc306 Δ <i>pthA4</i> , Gm | This study |
| Xcc306 Δ <i>pthA4</i> ::d <i>CsLOB4</i> /Xcc306 Δ <i>pthA4</i> ::d <i>CsLOB4-2</i> | Artificial TALE targeting <i>CsLOB4</i> complement Xcc306 Δ <i>pthA4</i> , Gm | This study |
| <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> Xcv8510 | Wild-type | Jeffery Johnes's laboratory (Gainesville, FL, USA) |
| Xcv8510::pHMI | Empty vector pHMI complement Xcv8510 | This study |
| Xcv8510::d <i>CsLOB2</i> | Artificial TALE targeting <i>CsLOB2</i> complement Xcv8510, Gm | This study |
| <i>Escherichia coli</i> DH5 α Plasmid | F--recA ϕ 80dlacZ Δ M15 | New England Biology (NEB) |
| pBluescript KS(+) | Phagemid, pUC derivative, Amp ^r | Stratagene |
| pHMI | Broad-host-range resistance to Sp | Hopkins <i>et al.</i> (1992) |
| pUFR053 | repW, Mob+, LacZ α +, Par+, Gmr | Clontech |

Amp^r, ampicillin-resistant; Gm, gentamicin; Sp, spectinomycin; Rif, rifamycin.

*DPI, Division of Plant Industry of the Florida Department of Agriculture and Consumer Services (Gainesville, FL, USA).

†BRL, Bethesda Research Laboratories (Gaithersburg, MD, USA).

suspended in sterilized double-distilled water to an optical density at 600 nm (OD₆₀₀) of 0.5 and, where required, the suspensions were diluted 10³ times in double-distilled water. The bacterial suspensions were delivered to the underside (abaxial) of young grapefruit leaves with a needleless 1-mL syringe.

Imaging of grapefruit leaves inoculated with various bacterial strains

Grapefruit leaves were removed from the trees when the phenotypes were ready for imaging; 1 \times photographs were taken using a digital SLR camera; 6.4 \times photographs were imaged using a Nikon SMZ800 dissecting microscope, which was equipped with a Nikon DS-Vi1 camera.

Phylogenetic analysis of *C. sinensis* LBD proteins

LBD protein sequences were downloaded from the Plant Transcription Factor Database (<http://planttfdb.cbi.pku.edu.cn/family.php?sp=Csi&fam=LBD>). Amino acid sequences were aligned using SeaView version 4 with the default parameters. The unrooted phylogenetic tree was computed using the setting of PhyML-3.1 with the LG amino acid substitution model and the non-parametric bootstrap support test. SeaView 4.1 was used to display and edit the phylogenetic trees (Gouy *et al.*, 2009).

Phylogenetic analysis of LBD proteins of *CsLOB1-4* and the closely related LBD members in *A. thaliana*

Sequences were aligned with the online CLUSTALW server (<http://www.ch.embnet.org/software/ClustalW.html>) using the default values. MEGA6.0 was used to generate a tree on the basis of CLUSTALW output. Phylogenetic calculations were based on the maximum likelihood method, and bootstrap analysis was used to evaluate the reliability of the nodes of the phylogenetic trees.

dTALE design and construction

Promoter sequences were the 1000-bp upstream DNA sequences from the initiation codon ATG of each *CsLOB* gene retrieved from the Phytozome database (http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Csinensis) according to the respective ID. The promoter regions of *CsLOB2*, *CsLOB3* and *CsLOB4* were scanned for potential EBEs using TAL Effector Nucleotide Targeter 2.0 (<https://tale-nt.cac.cornell.edu/node/add/talef-off>). Sites closest to the TATA box were selected as the targeted EBEs (Fig. S1A).

Four types of repeat (NI, NN, NG and HD), which correspond to the respective nucleotides A, G, T and C, were used to assemble the middle repeat domains of the artificial dTALEs according to the TAL effector assembly method of Li *et al.* (2011). The restriction enzymes *BstA*I and *Aat*II were used to replace the repeat region of the TAL effector gene *pthAw*. Each gene was sequenced to verify each construct.

Table 2 Genes whose log₂ fold changes are greater than two in a comparison between *Xcc306ΔpthA4::dCsLOB4* and *Xcc306ΔpthA4*, and the differential expression levels in three other comparisons: *Xcc306WT* vs. *Xcc306ΔpthA4*, *Xcc306ΔpthA4::dCsLOB2* vs. *Xcc306ΔpthA4*, and *Xcc306ΔpthA4::dCsLOB3* vs. *Xcc306ΔpthA4*.

| Gene ID (Phytozome) | log ₂ fold change (<i>Xcc306ΔpthA4::dCsLOB4</i> vs. <i>Xcc306ΔpthA4</i>) | log ₂ fold change (<i>Xcc306WT</i> vs. <i>Xcc306ΔpthA4</i>) | log ₂ fold change (<i>Xcc306ΔpthA4::dCsLOB2</i> vs. <i>Xcc306ΔpthA4</i>) | log ₂ fold change (<i>Xcc306ΔpthA4::dCsLOB3</i> vs. <i>Xcc306ΔpthA4</i>) |
|---------------------|---|--|---|---|
| orange1.1g036782m | Infinite | | | |
| orange1.1g011687m | 5.7325 | | | |
| orange1.1g048531m | 3.8539 | | | |
| orange1.1g023962m | 3.6642 | 2.8367 | 2.9486 | 6.5571 |
| orange1.1g040868m | 2.7413 | 2.7184 | 3.4253 | 6.2149 |
| orange1.1g040244m | 2.4699 | | 3.9254 | |
| orange1.1g034401m | 2.2665 | 1.8605 | 1.9224 | 4.3398 |
| orange1.1g038146m | 2.2480 | 2.6675 | 2.5362 | 4.7505 |
| orange1.1g007426m | 2.1797 | | 0.7902 | 4.7797 |
| orange1.1g044553m | 2.0393 | | 1.3147 | 2.2238 |

Blank cells indicate that the differential expression levels are not significant at a false discovery rate (FDR) < 0.05.

Infinite denotes that the fold change is positive infinite. The table is a derivative from the RNA-Seq analysis.

RNA extraction, reverse transcription and qRT-PCR analysis

Grapefruit leaves were syringe infiltrated with bacterial suspensions at OD₆₀₀ = 0.5, and the leaf tissues were harvested at 5 dpi. Total RNA was extracted using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. RNA was subjected to LiCl re-precipitation and DNase treatment to remove the remaining DNA. First-strand cDNA synthesis was achieved using a Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Two-step real-time PCR was performed using iQTM SYBR[®] Green Supermix (Bio-Rad, Hercules, CA, USA). The gene-specific primer sequences are listed in Table S2 (see Supporting Information). The gene with transcript ID number orange1.1g001725m (Phytozome) was used as an endogenous control. The 2^{-ΔΔCt} method was used for relative quantification (Livak and Schmittgen, 2001).

Bacterial population assay in citrus plants

Each strain was inoculated using a bacterial suspension solution of OD₆₀₀ = 0.5 diluted 10³ times in double-distilled water. Six 1-cm² pieces of leaf disc in the inoculation area for each treatment were taken and ground in 10 mL of sterile water; after serial dilution, 50 μL were plated on TSA medium and incubated at 28 °C for 2 days. The colony number was counted to determine the internal populations. Each treatment was repeated three times.

RNA-Seq assay and analysis

Two RNA-Seq assays were performed. For the first, three treatments of *Xcc306WT*, *Xcc306ΔpthA4* and *Xcc306ΔpthA4::dCsLOB2* with three replications for each strain were executed. For the second, three treatments of *Xcc306ΔpthA4*, *Xcc306ΔpthA4::dCsLOB3* and *Xcc306ΔpthA4::dCsLOB4* were performed, also with three replications for each strain. Libraries were constructed using the TruSeqTM RNA and DNA Sample Prep Kits (Illumina, San Diego, CA, USA). Nine samples were pooled into one lane for sequencing. For the first, Illumina High Output 100-cycle single-end (HO-

SR100) sequencing was used; for the second, an Illumina NextSeq500-MID Throughput 2 × 75 cycle run was performed.

The quality of raw reads was analysed with fastQC software (Andrews, 2010). Adapter trimming and filtering were conducted using FastX-toolKit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Reads were mapped to the reference genome sequence of *C. sinensis* obtained from the phytozome database V10 (<https://phytozome.jgi.doe.gov/pz/portal.html>) using TopHat V2.1 (Trapnell *et al.*, 2009). Gene expression quantification was conducted with HTSeq-count software (Anders *et al.*, 2015) using the gene annotation file from the *C. sinensis* genome project at Phytozome database v10. Differential gene expression analysis was conducted using DESeq (Anders and Huber, 2010). Genes were considered to be differentially expressed with a cutoff of the adjusted *P* value of 0.05. DEGs from both assays were compared to generate a Venn diagram using the downloaded software VennPlex version 1.0.0.2 (Cai *et al.*, 2013).

Gene annotation and enrichment were analysed using the online software Mercator employing the amino acid sequences with parameters all selected, except IS_DNA, and a BLAST_CUTOFF value was set as 50 (Lohse *et al.*, 2013).

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REFERENCES

- Alexandre, M., Do, A., Toledo, C.P., Baptista, J.C. and Machado, M.A. (2005) Transformation of *Xanthomonas axonopodis* pv. *citri* by electroporation. *Trop. Plant Pathol.* **30**, 292. doi:10.1590/S0100-41582005000300013.
- Al-Saadi, A., Reddy, J.D., Duan, Y.P., Brunings, A.M., Yuan, Q. and Gabriel, D.W. (2007) All five host-range variants of *Xanthomonas citri* carry one *pthA* homolog with 17.5 repeats on citrus, but none determine host-range pathogenicity variation. *Mol. Plant-Microbe Interact.* **20**, 934–943. doi:10.1094/MPMI-20-8-0934.
- Anders, S. and Huber, W. (2010) Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106. doi:10.1186/gb-2010-11-10-r106.

- Anders, S., Pyl, P.T. and Huber, W. (2015) HTSeq – a python framework to work with high-throughput sequencing data. *Bioinformatics*, **31**, 166–169. doi:10.1093/bioinformatics/btu638.
- Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data. Available at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.
- Antony, G., Zhou, J., Huang, S., Li, T., Liu, B., White, F. and Yang, B. (2010) Rice *xa13* recessive resistance to bacterial blight is defeated by induction of the disease susceptibility gene *Os11N3*. *Plant Cell*, **22**, 3864–3876. doi:10.1105/tpc.110.078964.
- Asano, K., Yamasaki, M., Takuno, S., Miura, K., Katagiri, S., Ito, T., Doi, K., Wu, J., Ebana, K., Matsumoto, T., Innan, H., Kitano, H., Ashikari, M. and Matsuoka, M. (2011) Artificial selection for a green revolution gene during japonica rice domestication. *Proc. Natl. Acad. Sci. USA*, **108**, 11 034–11 039. doi:10.1073/pnas.1019490108.
- Bellincampi, D., Cervone, F. and Lionetti, V. (2014) Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. *Front. Plant Sci.* **5**, 228. doi:10.3389/fpls.2014.00228.
- Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, and A., Bonas, U. (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. *Science*, **326**, 1509–1512. doi:10.1126/science.1178811.
- Boch, J., Bonas, U. and Lahaye, T. (2014) TAL effectors – pathogen strategies and plant resistance engineering. *New Phytol.* **204**, 823–832. doi:10.1111/nph.13015.
- Cai, H., Chen, H., Yi, T., Daimon, C.M., Boyle, J.P., Peers, C., Maudsley, S. and Martin, B. (2013) A novel Venn diagram program for comparing and visualizing datasets with differentially regulated datapoints. *PLoS One*, **8**, e53388. doi:10.1371/journal.pone.0053388.
- Cantu, D., Blanco-Ulate, B., Yang, L., Lavavitch, J.M., Bennett, A.B. and Powell, A.L.T. (2009) Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires NOR but not RIN or ethylene. *Plant Physiol.* **150**, 1434–1449. doi:10.1104/pp.109.138701.
- Cernadas, R.A., Doyle, E.L., Niño-Liu, D.O., Wilkins, K.E., Bancroft, T., Wang, L., Schmidt, C.L., Caldo, R., Yang, B., White, F.F., Nettleton, D., Wise, R.P., and Bogdanove, A.J. (2014) Code-assisted discovery of TAL effector targets in bacterial leaf streak of rice reveals contrast with bacterial blight and a novel susceptibility gene. *PLoS Pathog.* **10**, e1003972. doi:10.1371/journal.ppat.1003972.
- Chen, L., Hou, B., Lalonde, S., Takanaga, H., Hartung, M.L., Qu, X., Guo, W.J., Kim, J.G., Underwood, W., Chaudhuri, B., Chermak, D., Antony, G., White, F.F., Somerville, S.C., Mudgett, M.B. and Frommer, W.B. (2010) Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature*, **468**, 527–532. doi:10.1038/nature09606.
- Chen, L., Qu, X., Hou, B., Sosso, D., Osorio, S., Fernie, A.R. and Frommer, W.B. (2012) Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Science*, **335**, 207.
- Cohn, M., Bart, R.S., Shybut, M., Dahlbeck, D., Gomez, M., Morbitzer, R., Hou, B.H., Frommer, W.B., Lahaye, T. and Staskawicz, B.J. (2014) *Xanthomonas axonopodis* virulence is promoted by a transcription activator-like effector mediated induction of a SWEET sugar transporter in cassava. *Mol. Plant-Microbe Interact.* **27**(11), 1186–1198. doi:10.1094/MPMI-06-14-0161-R.
- De Feyter, R., Yang, Y. and Gabriel, D.W. (1993) Gene-for-gene interactions between cotton R genes and *Xanthomonas campestris* pv. *malvacearum* avr genes. *Mol. Plant-Microbe Interact.* **6**, 225.
- Flors, V., Leyva, M.D.L.O., Vicedo, B., Finiti, I., Real, M.D., Garcia-Agustin, P., Bennett, A.B. and González-Bosch, C. (2007) Absence of the endo-beta-1,4-glucanases Cel1 and Cel2 reduces susceptibility to *Botrytis cinerea* in tomato. *Plant J.* **52**, 1027–1040. doi:10.1111/j.1365-3113X.2007.03299.x.
- Garris, A., Tai, T., Coburn, J., Kresovich, S. and McCouch, S. (2005) Genetic structure and diversity in *Oryza sativa* L. *Genetics*, **169**, 1631–1638. doi:10.1534/genetics.104.035642.
- Gendron, J.M., Liu, J., Fan, M., Bai, M., Wenkel, S., Springer, P.S., Barton, M.K. and Wang, Z.Y. (2012) Brassinosteroids regulate organ boundary formation in the shoot apical meristem of *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **109**, 21 152–21 157. doi:10.1073/pnas.1210799110.
- Gent, D.H., Al-Saadi, A., Gabriel, D.W., Louws, F.J., Ishimaru, C.A. and Schwartz, H.F. (2005) Pathogenic and genetic relatedness among *Xanthomonas axonopodis* pv. *allii* and other pathovars of *X. axonopodis*. *Phytopathology*, **95**, 918–925. doi:10.1094/PHYTO-95-0918.
- Gottwald, T.R., Graham, J.H. and Schubert, T.S. (2002) Citrus canker: the pathogen and its impact. *Plant Health Prog.* **1**–32. doi:10.1094/PHP-2002-0812-01-RV.
- Gouy, M., Guindon, S. and Gascuel, O. (2009) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* **27**, 221–224. doi:10.1093/molbev/mps259.
- Grau, J., Wolf, A., Reschke, M., Bonas, U., Posch, S. and Boch, J. (2013) Computational predictions provide insights into the biology of TAL effector target sites. *PLoS Comput Biol.* **9**, e1002962. doi:10.1371/journal.pcbi.1002962.
- Heuer, H., Yin, Y., Xue, Q., Smalla, K. and Guo, J. (2007) Repeat domain diversity of avrBs3-like genes in *Ralstonia solanacearum* strains and association with host preferences in the field. *Appl. Environ. Microbiol.* **73**, 4379.
- Hopkins, C.M., White, F.F., Choi, S.H., Guo, A. and Leach, J.E. (1992) Identification of a family of avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant-Microbe Interact.* **5**, 451–459. doi:10.1094/MPMI-5-451.
- Hu, Y., Zhang, J., Jia, H., Sosso, D., Li, T., Frommer, W.B., Yang, B., White, F.F., Wang, N. and Jones, J.B. (2014) Lateral organ boundaries 1 is a disease susceptibility gene for citrus bacterial canker disease. *Proc. Natl. Acad. Sci. USA*, **111**, E521–E529. doi:10.1073/pnas.1313271111.
- Husbands, A., Bell, E.M., Shuai, B., Smith, H.M.S. and Springer, P.S. (2007) LATERAL ORGAN BOUNDARIES defines a new family of DNA-binding transcription factors and can interact with specific bHLH proteins. *Nucleic Acids Res.* **35**, 6663–6671. doi:10.1093/nar/gkm775.
- Hutin, M., Pérez-Quintero, A., Lopez, C. and Szurek, B. (2015) MorTAL combat: the story of defense against TAL effectors through loss-of-susceptibility. *Front. Plant Sci.* **6**. doi:10.3389/fpls.2015.00535.
- Jalan, N., Kumar, D., Andrade, M.O., Yu, F., Jones, J.B., Graham, J.H., White, F.F., Setubal, J.C. and Wang, N. (2013) Comparative genomic and transcriptome analyses of pathotypes of *Xanthomonas citri* subsp. *citri* provide insights into mechanisms of bacterial virulence and host range. *BMC Genomics*, **14**, 551. doi:10.1186/1471-2164-14-551.
- Kay, S., Hahn, S., Marois, E., Hause, G. and Bonas, U. (2007) A bacterial effector acts as a plant transcription factor and induces a cell size regulator. *Science*, **318**, 648–651. doi:10.1126/science.1144956.
- Khan, I.A. (2007) In: *Citrus Genetics, Breeding and Biotechnology* (Khan, I.A. ed.) pages 1–8. Wallingford, Oxfordshire: CAB International. doi:10.1079/9780851990194.000 ER.
- Li, T., Huang, S., Zhao, X., Wright, D.A., Carpenter, S., Spalding, M.H., Weeks, D.P. and Yang, B. (2011) Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. *Nucleic Acids Res.* **39**, 6315–6325. doi:10.1093/nar/gkr188.
- Li, T., Liu, B., Spalding, M.H., Weeks, D.P. and Yang, B. (2013) High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat. Biotechnol.* **30**, 390–392. doi:10.1038/nbt.2199.
- Li, T., Huang, S., Zhou, J. and Yang, B. (2013) Designer TAL effectors induce disease susceptibility and resistance to *Xanthomonas oryzae* pv. *oryzae* in rice. *Mol. Plant*, **6**, 781–789. doi:10.1093/mp/psst034.
- Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, **25**, 402–408. doi:10.1006/meth.2001.1262.
- Lohse, M., Nagel, A., Herter, T., May, P., Schroda, M., Zrenner, R., Tohge, T., Fernie, A.R., Stitt, M. and Usadel, B. (2013) Mercator: a fast and simple web server for genome scale functional annotation of plant sequence data. *Plant Cell Environ.* **37**, 1250–1258. doi:10.1111/pce.12231.
- Majer, C. and Hochholdinger, F. (2011) Defining the boundaries: structure and function of LOB domain proteins. *Trends Plant Sci.* **16**, 47–52. doi:10.1016/j.tplants.2010.09.009.
- Mak, A.N., Bradley, P., Bogdanove, A.J. and Stoddard, B.L. (2013) TAL effectors: function, structure, engineering and applications. *Curr. Opin. Struct. Biol.* **23**, 93–99. doi:10.1016/j.sbi.2012.11.001.
- Matsui, A., Ishida, J., Morosawa, T., Mochizuki, Y., Kaminuma, E., Endo, T.A., Okamoto, M., Nambara, E., Nakajima, M., Kawashima, M., Satou, M., Kim, J.M., Kobayashi, N., Toyoda, T., Shinozaki, K. and Seki, M. (2008) Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant Cell Physiol.* **49**, 1135–1149. doi:10.1093/pcpp/pcn101.
- Morbitzer, R., Roemer, P., Boch, J. and Lahaye, T. (2010) Regulation of selected genome loci using de novo-engineered transcription activator-like effector (TALE)-type transcription factors. *Proc. Natl. Acad. Sci. USA*, **107**, 21 617–21 622. doi:10.1073/pnas.1013133107.
- Moscou, M.J. and Bogdanove, A.J. (2009) A simple cipher governs DNA recognition by TAL effectors. *Science*, **326**, 1501. doi:10.1126/science.1178817.
- Nakazawa, M., Ichikawa, T., Ishikawa, A., Kobayashi, H., Tshara, Y., Kawashima, M., Suzuki, K., Muto, S. and Matsui, M. (2003) Activation tagging, a novel tool to dissect the functions of a gene family. *Plant J.* **34**, 741–750. doi:10.1046/j.1365-3113X.2003.01758.x.

- Pereira, A.L.A., Carazzolle, M.F., Abe, V.Y., de Oliveira, M.L.P., Domingues, M.N., Silva, J.C., Cernadas, R.A. and Benedetti, C.E. (2014) Identification of putative TAL effector targets of the citrus canker pathogens shows functional convergence underlying disease development and defense response. *BMC Genomics*, **15**, 157. doi:10.1186/1471-2164-15-157.
- Salzberg, S.L., Sommer, D.D., Schatz, M.C., Phillippy, A.M., Rabinowicz, P.D., Tsuge, S., Furutani, A., Ochiai, H., Delcher, A.L., Kelley, D., Madupu, R., Puiu, D., Radune, D., Shumway, M., Trapnell, C., Aparna, G., Jha, G., Pandey, A., Patil, P.B., Ishihara, H., Meyer, D.F. and Szurek, B. (2008) Genome sequence and rapid evolution of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* PXO99A. *BMC Genomics*, **9**, 534. doi:10.1186/1471-2164-9-534.
- Shinada, H., Yamamoto, T., Yamamoto, E., Hori, K., Yonemaru, J., Matsuba, S. and Fujino, K. (2014) Historical changes in population structure during rice breeding programs in the northern limits of rice cultivation. *Theor. Appl. Genet.* **127**, 995–1004. doi:10.1007/s00122-014-2274-2.
- Shuai, B., Reynaga-Pena, C.G. and Springer, P.S. (2002) The LATERAL ORGAN BOUNDARIES gene defines a novel, plant-specific gene family. *Plant Physiol.* **129**, 747–761. doi:10.1104/pp.010926.
- da Silva, A.C., Ferro, J.A., Reinach, F.C., Farah, C.S., Furlan, L.R., Quaggio, R.B., Monteiro-Vitorello, C.B., Van Sluys, M.A., Almeida, N.F., Alves, L.M., do Amaral, A.M., Bertolini, M.C., Camargo, L.E., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L.P., Cicarelli, R.M., Coutinho, L.L., Cursino-Santos, J.R., El-Dorry, H., Faria, J.B., Ferreira, A.J., Ferreira, R.C., Ferro, M.I., Formighieri, E.F., Franco, M.C., Greggio, C.C., Gruber, A., Katsuyama, A.M., Kishi, L.T., Leite, R.P., Lemos, E.G., Lemos, M.V., Locali, E.C., Machado, M.A., Madeira, A.M., Martinez-Rossi, N.M., Martins, E.C., Meidanis, J., Menck, C.F., Miyaki, C.Y., Moon, D.H., Moreira, L.M., Novo, M.T., Okura, V.K., Oliveira, M.C., Oliveira, V.R., Pereira, H.A., Rossi, A., Sena, J.A., Silva, C., de Souza, R.F., Spinola, L.A., Takita, M.A., Tamura, R.E., Teixeira, E.C., Tezza, R.I., Trindade dos Santos, M., Truffi, D., Tsai, S.M., White, F.F., Setubal, J.C. and Kitajima, J.P. (2002) Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature*, **417**, 459–463. doi:10.1038/417459a.
- Streubel, J., Pesce, C., Hutin, M., Koebnik, R., Boch, J. and Szurek, B. (2013) Five phylogenetically close rice SWEET genes confer TAL effector-mediated susceptibility to *Xanthomonas oryzae* pv. *oryzae*. *New Phytol.* **200**, 808–819. doi:10.1111/nph.12411.
- Sun, X.A., Stall, R.E., Jones, J.B., Cubero, J., Gottwald, T.R., Graham, J.H., Dixon, W.N., Schubert, T.S., Chaloux, P.H., Stromberg, V.K., Lacy, G.H. and Sutton, B.D. (2004) Detection and characterization of a new strain of citrus canker bacteria from key Mexican lime and alewom in south Florida. *Plant Dis.* **88**, 1179–1188. doi:10.1094/PDIS.2004.88.11.1179.
- Swarup, S., De Feyter, R., Brlansky, R.H. and Gabriel, D.W. (1991) A pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *X. campestris* to elicit cankerlike lesions on citrus. *Phytopathology*, **81**, 802–809. doi:10.1094/Phyto-81-802.
- Swarup, S., Yang, Y.N., Kingsley, M.T. and Gabriel, D.W. (1992) An *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhosts. *Mol. Plant–Microbe Interact.* **5**, 204–213. doi:10.1094/MPMI-5-204.
- Thatcher, L.F., Powell, J.J., Aitken, E.A.B., Kazan, K. and Manners, J.M. (2012) The lateral organ boundaries domain transcription factor LBD20 functions in fusarium wilt susceptibility and jasmonate signaling in *Arabidopsis*. *Plant Physiol.* **160**, 407–418. doi:10.1104/pp.112.199067.
- Trapnell, C., Pachter, L. and Salzberg, S.L. (2009) TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics*, **25**, 1105–1111. doi:10.1093/bioinformatics/btp120.
- Verniere, C., Hartung, J.S., Pruvost, O.P., Civerolo, E.L., Alvarez, A.M., Maestri, P. and Luisetti, J. (1998) Characterization of phenotypically distinct strains of *Xanthomonas axonopodis* pv. *citri* from southwest Asia. *Eur. J. Plant Pathol.* **104**, 477–487. doi:10.1023/A:1008676508688.
- Wang, X., Zhang, S., Su, L., Liu, X. and Hao, Y. (2013) A genome-wide analysis of the LBD (LATERAL ORGAN BOUNDARIES domain) gene family in *Malus domestica* with a functional characterization of MdLBD11. *PLoS One*, **8**, e57044. doi:10.1371/journal.pone.0057044.
- Yang, B., Sugio, A. and White, F.F. (2006) Os8N3 is a host disease-susceptibility gene for bacterial blight of rice. *Proc. Natl. Acad. Sci. USA*, **103**, 10 503–10 508. doi:10.1073/pnas.0604088103.
- Zhang, J., Yin, Z. and White, F. (2015) TAL effectors and the executor R genes. *Front. Plant Sci.* **6**, 641. doi:10.3389/fpls.2015.00641.
- Zhou, J., Peng, Z., Long, J., Sosso, D., Liu, B., Eom, J., Huang, S., Liu, S., Vera Cruz, C., Frommer, W.B., White, F.F. and Yang, B. (2015) Gene targeting by the TAL effector PthXo2 reveals cryptic resistance gene for bacterial blight of rice. *Plant J.* **82**, 632–643. doi:10.1111/tj.12838.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

File S1 The accession ID (Phytozome) and protein sequences of the 34 lateral organ boundary domain (LBD) members in *Citrus sinensis* downloaded from the Plant Transcription Factor Database (<http://plantfdb.cbi.pku.edu.cn/family.php?sp=Csi&fam=LBD>).

File S2 Mercator analysis of the genes that were up-regulated by Xcc306WT, Xcc306ΔpthA4::dCsLOB2 and Xcc306ΔpthA4::dCsLOB3 relative to Xcc306ΔpthA4. A log₂(fold change) larger than unity was applied as the filtering threshold.

File S3 Mercator analysis of the genes that were down-regulated by Xcc306WT, Xcc306ΔpthA4::dCsLOB2 and Xcc306ΔpthA4::dCsLOB3 relative to Xcc306ΔpthA4. A log₂(fold change) smaller than −1 was applied as the filtering threshold.

File S4 RNA-sequencing (RNA-Seq) analysis results of genes that are commonly regulated by Xcc306WT, Xcc306ΔpthA4::dCsLOB2, Xcc306ΔpthA4::dCsLOB3 and Xcc306ΔpthA4::dCsLOB4 relative to Xcc306ΔpthA4.

Fig. S1 Schematic diagrams of designed transcription activator-like effectors (dTAles) for CsLOB2, CsLOB3 and CsLOB4. (a) Promoter regions of three targeted lateral organ boundary domain (LBD) genes in *Citrus sinensis*. The yellow highlight shows the effector-binding elements (EBEs) targeted by dTAles, and the red letters indicate the predicted TATA boxes and start codons for each gene. (b) The repeat variable diresidues (RVDs) of three dTAles and the corresponding EBEs in the promoters of CsLOB2, CsLOB3 and CsLOB4. (c) The transcription activator-like (TAL) code based on Grau *et al.* (2013).

Fig. S2 dCsLOB2 induces CsLOB2 expression when transformed into *Xanthomonas euvesicatoria* strain Xcv85-10. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) of RNA samples of grapefruit leaves inoculated with Xcv85-10::pHM1 and Xcv85-10::dCsLOB2 [5 days post-inoculation (dpi)]. Inoculum was a bacterial suspension with an optical density at 600 nm (OD₆₀₀) of 0.5. Samples were taken at 5 dpi. Error bars represent the standard errors for three independent experimental replicates. *Significant difference between the two treatments (*t*-test, *P* < 0.05).

Fig. S3 Functional category assignments of common genes that were significantly regulated in grapefruit by Xcc306WT, Xcc306ΔpthA4::dCsLOB2 and Xcc306ΔpthA4::dCsLOB3 relative

to *Xcc306ΔpthA4*. (a) Mercator analysis of the up-regulated genes was performed using the 466 genes related to the expression of *CsLOB1*, *CsLOB2* and *CsLOB3* in grapefruit at 5 days post-inoculation (dpi). A \log_2 (fold change) larger than unity was applied as the filtering threshold. (b) Mercator analysis of the down-regulated genes was performed using the 564 genes related to the expression of *CsLOB1*, *CsLOB2* and *CsLOB3* in grapefruit at 5 dpi. A \log_2 (fold change) smaller than -1 was applied as the infiltration threshold.

Fig. S4 Phylogenetic analysis of lateral organ boundary domain (LBD) proteins of *CsLOB1*, *CsLOB2*, *CsLOB3*, *CsLOB4* and closely related LBD members in *Arabidopsis thaliana*. (a) Phylogenetic tree of LBD proteins of *CsLOB1*, *CsLOB2*, *CsLOB3*, *CsLOB4* and closely related LBD members in *A. thaliana*. A monophyletic group containing *CsLOB1*, *CsLOB2* and *CsLOB3* is boxed in red. The group containing *CsLOB4* is boxed in blue. Bootstrap values were based on 500 replications. The branch lengths of the tree are proportional to divergence. The 0.1 scale represents 10% change. (b) Protein sequence alignment of LBD genes from *Citrus sinensis* (Cs) and *Arabidopsis thaliana* (At). Three conserved motifs (CX2CX6CX3C, glycine residue and LX6LX3LX6L) are highlighted in red, pink and blue, respectively. The alignment was conducted using CLUSTALW with the default settings.

Fig. S5 A second designed transcription activator-like effector (dTALe) targeting *CsLOB4* did not induce pustule formation on citrus. (a) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) validation of *CsLOB4* induction by d*CsLOB4-2*. Asterisk denotes a significant difference in the fold change between the treatments relative to the negative control *Xcc306ΔpthA4* at $P < 0.05$ using *t*-test. qRT-PCR was conducted on mRNA extracted from grapefruit leaves inoculated with the individual *Xanthomonas citri* ssp. *citri* (Xcc) strains as

indicated, using *CsLOB4* gene-specific primers at 5 days post-inoculation (dpi). Expression values were normalized to the housekeeping gene orange1.1g001725m. Error bars represent the standard deviations for three independent experimental replicates. Samples were taken at 5 dpi. (b) Pustule was not induced by the two dTALes targeting *CsLOB4*: 1, *Xcc306ΔpthA4*; 2, *Xcc306ΔpthA4::dCsLOB4*; 3, *Xcc306ΔpthA4::dCsLOB4-2*. Photographs were taken at 7 dpi of various strains as shown in the figures. Inocula were bacterial suspensions with an optical density at 600 nm (OD_{600}) of 0.5.

Fig. S6 A second longer version of designed transcription activator-like effector (dTALe) targeting *CsLOB2* induced pustule formation on citrus. (a) Pustule was induced by the two dTALes targeting *CsLOB2*: 1, *Xcc306ΔpthA4::dCsLOB2L*; 2, *Xcc306ΔpthA4*. Photographs were taken at 7 days post-inoculation (dpi) of various strains as shown in the figures. Inocula were bacterial suspensions with an optical density at 600 nm (OD_{600}) of 0.5. (b) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) validation of *CsLOB2* induction by d*CsLOB2L*. Asterisk denotes significant difference in the fold change between the treatments relative to the negative control *Xcc306ΔpthA4* at $P < 0.05$ using *t*-test. qRT-PCR was conducted on mRNA extracted from grapefruit leaves inoculated with the individual *Xanthomonas citri* ssp. *citri* (Xcc) strains as indicated, using *CsLOB2* gene-specific primers at 5 dpi. Expression values were normalized to the housekeeping gene orange1.1g001725m. Error bars represent the standard deviations for three independent experimental replicates. Samples were taken at 5 dpi.

Table S1 Cell wall-related genes clustered by Mercator analysis. The yellow highlighted genes are those tested by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Table S2 Primers used in this study