Genome editing of the disease susceptibility gene CsLOB1 in citrus confers resistance to citrus canker

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Abstract

Citrus is a highly valued tree crop worldwide, while, at the same time, citrus production faces many biotic challenges, including bacterial canker and Huanglongbing (HLB). Breeding for disease-resistant varieties is the most efficient and sustainable approach to control plant diseases. Traditional breeding of citrus varieties is challenging due to multiple limitations, including polyploidy, polyembryony, extended juvenility and long crossing cycles. Targeted genome editing technology has the potential to shorten varietal development for some traits, including disease resistance. Here, we used CRISPR/Cas9/sgRNA technology to modify the canker susceptibility gene CsLOB1 in Duncan grapefruit. Six independent lines, DLOB2, DLOB3, DLOB9, DLOB10, DLOB11 and DLOB12, were generated. Targeted next-generation sequencing of the six lines showed the mutation rate was 31.58%, 23.80%, 89.36%, 88.79%, 46.91% and 51.12% for DLOB2, DLOB3, DLOB9, DLOB10, DLOB11 and DLOB12, respectively, of the cells in each line. DLOB2 and DLOB3 showed canker symptoms similar to wild-type grapefruit, when inoculated with the pathogen Xanthomonas citri subsp. citri (Xcc). No canker symptoms were observed on DLOB9, DLOB10, DLOB11 and DLOB12 at 4 days postinoculation (DPI) with Xcc. Pustules caused by Xcc were observed on DLOB9, DLOB10, DLOB11 and DLOB12 in later stages, which were much reduced compared to that on wild-type grapefruit. The pustules on DLOB9 and DLOB10 did not develop into typical canker symptoms. No side effects and off-target mutations were detected in the modified grapefruit lines. This study indicates that genome editing using CRISPR technology will provide a promising pathway to generate disease-resistant citrus varieties.

Keywords: Xanthomonas citri, Cas9, sgRNA, Citrus paradisi.

Introduction

Citrus varieties are high value tree crops with plantings in over one hundred countries. The fruit provides numerous benefits to human, including providing vitamins, fibre, calcium, potassium, folate and lowering health risks. Citrus production faces many biotic and abiotic challenges. Among them, the bacterial pathogens Xanthomonas citri ssp. citri (Xcc) and Candidatus Liberibacter asiaticus are the causal agents for citrus canker and HLB disease, respectively. Breeding disease-resistant varieties is the most efficient and sustainable approach to control plant diseases. However, traditional citrus breeding has often been hindered by polyploidy, pollen-ovule sterility, sexual and graft incompatibilities, and extended juvenility (Davey et al., 2005). Various biotechnology methods have been used to develop modified and novel citrus varieties (Chen et al., 2013; Dutt et al., 2015; Fu et al., 2011). However, no genetically modified varieties have been commercialized. The lack of commercial releases has been attributed to the lack of consumer acceptance of transgene technology. Recent developments in targeted genome editing technologies, however, have facilitated the process to establish genetically modified cultivars that lack transgenes in the final line (Doudna and Charpentier, 2014).

We previously identified CsLOB1 as a critical citrus disease susceptibility gene for citrus canker (Hu et al., 2014). CsLOB1 is a member of the Lateral Organ Boundaries Domain (LBD) gene family of plant transcription factors. All strains of Xcc and a related pathogen X. fuscans subsp. aurantifolii (Xfa) encode transcription activator-like (TAL) effectors that recognize an effector binding element (EBE) in the promoter of CsLOB1 and induce expression of the disease susceptibility gene (Hu et al., 2014). Furthermore, the EBEs of individual critical TAL effectors in various canker-causing strains overlap (Hu et al., 2014). Thus, the EBE region of CsLOB1 may be the Achilles’ heel of citrus canker and presents an attractive target for genomic engineering of broad resistance to citrus canker. Previously, genome modifications of EBE regions of susceptibility genes Os11N3, Os14N3 and Os12N3 (also called OsSWEET14, OsSWEET11 and OsSWEET13, respectively) have generated resistance in rice to bacterial blight, which is incited by X. oryzae pv. oryzae using a set of TAL effector genes related to the critical TAL effectors of Xcc and Xga (Blanvillain-Baufumé et al., 2016; Li et al., 2012; Zhou et al., 2015). In our recent study, genome modification of the EBE of one single allele of CsLOB1 gene in grapefruit Duncan (Citrus paradisi Macf.) alleviated the canker symptoms due to a specific TAL effector (Jia et al., 2016). However, the modified grapefruit line, which is a hybrid, is still susceptible to wild-type Xcc as only one CsLOB1 allele was altered, and mutation of the EBEs of both alleles of CsLOB1 is required to generate reduced symptom plants (Jia et al., 2016). Here, we...
reported our progress to generate canker-resistant citrus by disrupting the coding region of both alleles of CsLOB1 using Cas9/sgRNA.

Results

We first targeted the CsLOB1 coding region using Cas9/sgRNA in a transient assay on Duncan grapefruit (Citrus × paradisi), as grapefruit is one of the most canker susceptible citrus varieties. Grapefruit contains two alleles of CsLOB1, Type I and Type II (Jia et al., 2016) resulting from grapefruit being a hybrid of maternal donor pummelo (C. maxima) and paternal donor sweet orange (C. sinensis) (Velasco and Licciardello, 2014) (Figure 1). The two alleles of CsLOB1 showed polymorphisms at both nucleotide and protein levels. The sgRNA was selected to target a conserved region of the 1st exon in both alleles

| Type   | Sequence                                                                 | Length |
|--------|--------------------------------------------------------------------------|--------|
| Type I | ATTGTCATTCCTGCTTCTTTTCTCTCTCTATATAAACCCCTTTTGGCTTGAACTTTGTTTC            | 60     |
| Type II| ATTGTCATTCCTGCTTCTTTTCTCTCTCTATATAAACCCCTTTTGGCTTGAACTTTGTTTC           | 59     |
| Type I | AACTAAAGCAGCTCTCCTCCTCCCTAACTGCTTTGCTTTCTCACTAACTACTACAACC              | 120    |
| Type II| AACTAAAGCAGCTCTCCTCCTCCCTAACTGCTTTGCTTTCTCACTAACTACTACAACC             | 119    |
| Type I | CAACAGTTTTCTCTCTCAAAATGGAATGCAAACACAAAATTAATGTAGCAATCCCAAT              | 180    |
| Type II| CAACAGTTTTCTCTCTCAAAATGGAATGCAAACACAAAATTAATGTAGCAATCCCAAT             | 179    |
| Type I | TCACACCTCTCCACGTCTCCCTCTCTCCTCAATCATCAAAATGGAATGCAAACACAAAATTAATGTAGCAAT | 240    |
| Type II| TCACACCTCTCCACGTCTCCCTCTCTCCTCAATCATCAAAATGGAATGCAAACACAAAATTAATGTAGCAAT | 239    |
| Type I | TCCAAGCTTTAAAGCTTCTCCTCTCTCCTCAATGGAATGCAAACACAAAATTAATGTAGCAATCCCAATCTCTCC | 360    |
| Type II| TCCAAGCTTTAAAGCTTCTCCTCTCTCCTCAATGGAATGCAAACACAAAATTAATGTAGCAATCTCTCC | 359    |
| Type I | GCCGCCCTATAGTCTTAGCTGCTCTGGCAAAATGGAATGCCCTCGCCGAGATGCTGTCGA             | 420    |
| Type II| GCCGCCCTATAGTCTTAGCTGCTCTGGCAAAATGGAATGCCCTCGCCGAGATGCTGTCGA            | 419    |
| Type I | GAAATGTGTTTTAGCTTCAAACTTTTCCACCAACCAGGACCATATGCATACCATTGCTCA            | 480    |
| Type II| GAAATGTGTTTTAGCTTCAAACTTTTCCACCAACCAGGACCATATGCATACCATTGCTCA           | 479    |
| Type I | TAGGGCTCCCTGCTGCTACCAATATCATCAAAATGTAGCAATCCCTCTCCTCTCCTCTCCTCCTCC     | 540    |
| Type II| TAGGGCTCCCTGCTGCTACCAATATCATCAAAATGTAGCAATCCCTCTCCTCTCCTCTCCTCCTCC    | 539    |
| Type I | TGAAATATCAAACCTTAAATTGTCACAAAACCAACCAACCGAATTGTAACCACATGCACTACCATTGCTCA | 599    |
| Type II| TGAAATATCAAACCTTAAATTGTCACAAAACCAACCAACCGAATTGTAACCACATGCACTACCATTGCTCA | 599    |
| Type I | AACTTGTTAATTGTTTTATTTTCATCAATTAGTTGTGTGATTAGACTTTGGAGTGGTTGA           | 659    |
| Type II| AACTTGTTAATTGTTTTATTTTCATCAATTAGTTGTGTGATTAGACTTTGGAGTGGTTGA          | 659    |
| Type I | TTTTCCGACTTTTCTTTTGGAAATCTGCGACTTCTCTACAAATGGAATGCAAACACAAAATTAATGTAGCAAT | 719    |
| Type II| TTTTCCGACTTTTCTTTTGGAAATCTGCGACTTCTCTACAAATGGAATGCAAACACAAAATTAATGTAGCAAT | 719    |

Figure 1 Alignment of Type I CsLOB1 and Type II CsLOB1 in Duncan grapefruit. Two alleles of CsLOB1, Type I and Type II, are present in Duncan grapefruit. Part of the promoter regions and coding sequences are shown, in which the difference was indicated by purple, and the PthA4 effector binding elements were highlighted by blue. The intron was highlighted in grey. The translation start site was highlighted in green. The sgRNA-targeting region, which is conservative on both alleles, was highlighted in red. The primers were underlined, which were used to analyse indel mutation in genome-modified Duncan by targeted next-generation sequencing.
(Figures 1 and S1). To facilitate the screen process, a binary vector GFP-p1380N-Cas9/sgRNA:cslob1, which contains a GFP reporter gene, was constructed (Figure S1). Citrus plants transformed with GFP-p1380N-Cas9/sgRNA:cslob could be readily monitored with GFP fluorescence. First, Xcc-facilitated Agrobacterium-mediated infiltration (Jia and Wang, 2014) and transient expression in citrus leaves were used to test GFP-p1380N-Cas9/sgRNA:cslob1 function. Four days after infiltration, GFP fluorescence was observed at the inoculation site, whereas no GFP signal was observed at the site of infiltration with the control vector p1380-AtHSP70BP-GUSin (Jia and Wang, 2014) (Figure 2a). PCR amplification and sequencing confirmed the targeted modification of CsLOB1 (Figure 2b and c). Therefore, GFP-p1380N-Cas9/sgRNA:cslob1 is functional for CsLOB1 coding region targeting.

Via Agrobacterium-mediated transformation, Duncan grapefruit epicotyls were used as explants to create transgenic citrus plants (Orbović and Grosser, 2015). Six independent transgenic lines, DLOB2, DLOB3, DLOB9, DLOB10, DLOB11 and DLOB12, were selected based on GFP fluorescence and verified by PCR analyses (Figure 3a and b). To calculate the mutation frequency and determine the genotype for CsLOB1 locus, targeted next-generation sequencing of the six transgenic lines was performed on amplified fragments using primers targeting a 380-bp region cover the targeted site. In total, more than 50 000 paired-end reads were generated for each sample, and, after filtering and quality trimming, the reads were grouped to clusters with a threshold of 100% pairwise identity using UCLUST (Edgar, 2010) (Table S1). Based on the sequencing results, the mutation rate was 31.58%, 23.80%, 89.36%, 88.79%, 46.91% and 51.12%
for DLOB2, DLOB3, DLOB9, DLOB10, DLOB11 and DLOB12, respectively (Figure 4a, Table S2). More than half of the mutations were 1-bp insertions of A or T, resulting in frame shift (Figure 4b, Table S2). The majority of deletions were short, ranging from 2 bp to 22 bps. Most of the 2-bp deletions were GA deletions (Figure 4b and Table S2). The 1-bp insertions took place at the 4th bp upstream of the PAM site (Figure 4b). The GA and GAGA deletions also occurred three base pairs upstream of the PAM site (Figure 4b), which is consistent with the previous report that Cas9 nuclease cleaves target DNA at a position three base pairs upstream of the PAM sequence (Jinek et al., 2012). Lines DLOB9 and DLOB10 were further confirmed using Sanger sequencing analysis (Figure S2).

The susceptibility of the six CsLOB1-modified Duncan grapefruit plants was tested by challenging with Xcc at the concentration of 5 x 10^6 CFU/mL. DLOB2 and DLOB3 showed canker symptoms similar to wild-type Duncan grapefruit. No canker symptoms were observed on DLOB9, DLOB10, DLOB11 and DLOB12 at 4 DPI (Figure 3c). Pustules caused by Xcc were observed on DLOB9, DLOB10, DLOB11 and DLOB12 in later stages, which were much reduced compared to that on wild-type grapefruit. The pustules on DLOB9 and DLOB10 did not develop into typical canker symptoms, whereas reduced canker symptoms were observed on DLOB11 and DLOB12 (Figure S3). The appearance of the pustules on DLOB9 and DLOB10 might result from wild-type cells or mutants that could not abolish the CsLOB1 function. We did not observe any visible phenotype change for CsLOB1-edited grapefruit lines (Figure S4).

We analysed potential off-target mutagenesis. Seven potential off-target sequences were identified from genomic data (Table S3). No off-target mutations in amplified fragments encompassing the sites were identified in the six CsLOB1-modified plants (Table S3). Due to the somatic nature of the plants and only ten random colonies per putative off-target site were sequenced, the possibility of off-target changes in a fraction of the cells cannot be ruled out.

**Discussion**

This study tested the proof of concept that we can generate canker-resistant plants by modifying susceptibility gene CsLOB1. In this study, the mutation rate was 89.36% and 88.79% for DLOB9 and DLOB10, respectively, and both lines showed canker resistance (Figure 3c), even though pustules can be observed at later stage (Figure 3). The mutation rate for DLOB11 and DLOB12 was 46.91% and 51.12%, respectively. Both DLOB11 and DLOB12 showed enhanced resistance against Xcc with more pustules than DLOB9 and DLOB10 (Figures 3c, S3). On the other hand, the

**Figure 4** Indel mutation rates and mutation genotypes in six CsLOB1-edited grapefruit lines. (a) Mutation rate for each CsLOB1-edited grapefruit line. Targeted next-generation sequencing was conducted for each line, and more than 50 000 paired-end reads were generated for each sample. (b) Representative indel mutation genotypes in Type I CsLOB1 plus Type II CsLOB1. The mutations included 1-bp insertion and short deletions. It should be noted that the AGAGAGGGGAG/G(CT)CTGCA deletion and GGAGAGGGGAG/G(CT)CTGCAAGATTT deletion removed the PAM and the SNP nucleotide. Star indicates SNP (single nucleotide polymorphism) used for differentiating type I and type II alleles of CsLOB1. 

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mutation rate for DLOB2 and DLOB3 was 31.58% and 23.80%, respectively. Neither DLOB2 nor DLOB3 showed resistance to citrus canker (Figures 3c, S3). The appearance of the pustules on DLOB9, DLOB10, DLOB11 and DLOB12 at 7 DPI might result from wild-type cells or mutants that could not abolish the CsLOB1 function. This recessive resistance due to mutation of CsLOB1 is expected to be durable and efficient against all Xanthomonas pathotypes causing citrus canker because they all rely on induction of the susceptibility gene CsLOB1 to induce canker symptoms. This is consistent with previous studies that mutation of the coding region of susceptibility gene will lead to disease resistance. Mutation of the susceptibility gene OsSWEET13 corresponding to PthXo2 of X. oryzae pv. oryzae has generated disease-resistant rice (Zhou et al., 2015). We need to point out that the genome-modified citrus lines are not suitable for application to control citrus canker at this moment as they still contain Cas9 and sgRNA in the genome, thus are considered as transgenic, and require rigorous registration process before allowed for commercialization. Recently, USDA has granted nonregulatory status for the genome modified common white button mushroom (Agaricus bisporus) resisting against browning (Waltz, 2016b) and high amylopectin corn generated by knocking out the endogenous waxy gene Wx1 (Waltz, 2016a) because both do not contain foreign DNAs and are without off-target mutations. Consequently, we need to generate nontransgenic canker-resistant citrus varieties to facilitate the de-regulation.

No phenotypic changes were observed for the CsLOB1-modified plants. CsLOB1 belongs to the LBD proteins which are transcription factors in the regulation of plant growth and development (Hudens et al., 2007). The biological function of CsLOB1 remains to be determined. RNA-Seq analysis of the expression profiles associated with CsLOB1 identified many downstream genes, for example cell organization, cell division, cell cycle, cell wall degradation and cell wall modification (Zhang et al., 2016). PtalBD1 derived from populus is a homolog of CsLOB1. It was reported that PtalBD1 was involved in secondary woody growth in poplar (Yordanov et al., 2010). The expression of PtalBD1-SRDX, harnessed for dominant-negative suppression of PtalBD1, suppressed stem diameter growth. No phenotypic changes observed for the CsLOB1-modified plants are probably due to the fact that citrus contains multiple LOB genes with similar functions, for example CsLOB1, CsLOB2 and CsLOB3. Induction of CsLOB2 and CsLOB3 using custom-designed TAL effectors leads to similar canker symptoms due to induction of CsLOB1 by PthA4 (Zhang et al., 2016), indicating similar functions of CsLOB1, CsLOB2 and CsLOB3. Thus, redundancy of CsLOB1 might be the main reason for the lack of phenotypic effect of the mutation. No off-target mutations were observed in the genome-modified plants, which might also contribute to the lack of phenotypic effect of the mutation. We could not totally rule out other phenotypic effects, for example flowering, as the genome-modified lines will take 2 to 3 more years to flower.

In summary, we have shown that mutation of the coding region of both alleles of the susceptibility gene CsLOB1 can generate citrus canker-resistant plants. Future work needs to focus on generating CsLOB1-modified citrus varieties which do not contain foreign DNA and comprise no off-target mutations for application purpose. Importantly, this study showed that we can generate disease-resistant citrus varieties using CRISPR technology and provide a long-term and efficient control measurement for other citrus diseases including HLB.

Materials and methods

Plasmid construction

The CaMV 35S promoter was amplified using primers CaMV35-5'-Xhol (5'-AAGCTTCAATAAGCTTACACAG-3') and sgRNA-cslob1-P1 (5'-phosphorylated-TATATGCTCTCCCTAA-3'). The CaMV 35S terminator was amplified using primers CsVMV-3'-SpeI (5'-AGGTAGCTTACACAG-3') and sgRNA-cslob1-P1 (5'-AGGTAGCTTACACAG-3') from plasmid p1380N-Cas9 to form p1380N-Cas9-35S-35S-gfp. The cassava vein mosaic virus promoter (CsVMV) was amplified using primers CsVMV-GFP-spt (5'-AAGCTTACACAG-3') and sgRNA-cslob1-P1 (5'-AGGTAGCTTACACAG-3') and sgRNA-NosT fragment was amplified using primers 35T-P1 (5'-GGCGGCGGATCTAGTAACATA-3') and 35T-P2 were used to amplify the DNA fragments from genomic DNA was extracted from wild-type Duncan, or transgenic plants, or the GFP-positive Duncan leaves treated by Xhol digested CaMV35S and Ascl-cut sgRNA-NosT were inserted into Xhol-Ascl-treated p1380N-Cas9 to form p1380N-Cas9/sgrna:cslob1. The p1380N-Cas9 was described previously (Jia and Wang, 2014).

Using a pair of primers 35T-P1 (5'-AGGTGAGATCCGAGTCTTTCATTTTCAATATATAATAAGGATAATAC-3') and 35T-P2 (5'-AGGTGAGATCCGAGTCTTTCATTTTCAATATATAATAAGGATAATAC-3'), GGTGAGATCCGAGTCTTTCATTTTCAATATATAATAAGGATAATAC-3' and GGTGAGATCCGAGTCTTTCATTTTCAATATATAATAAGGATAATAC-3' were used to amplify the target sequence. Recombinant Agrobacterium cells were employed for citrus transformation or Xcc-facilitated agroinfiltration.

Duncan CsLOB1 sequencing and analysis

Using a Wizard Genomic DNA Purification Kit (Promega), genomic DNA was extracted from wild-type Duncan, or transgenic plants, or the GFP-positive Duncan leaves treated by Xanthomonas citri sp. citri (Xcc)-facilitated agroinfiltration of GFP-p1380N-Cas9/sgrna:cslob1 (Figure 3a). To analyse CsLOB1 gene in detail, PCR was performed with the Phusion DNA polymerase (New England Biolabs) and a pair of primers, CsLBDP-5-P1 (5'-ATTGTCACTTCTGCTTCCTCTTCTCTCTCC-3') and CsLBDP-3-P2 (5'-TCAGTTGAATGTACACTCTCCTTCTCTCTCC-3'), flanking part of CsLOB1 promoter and its coding region. By blunt end cloning, the PCR products were inserted into the PCR-BluntII-TOPO vector (Life Technologies). The colonies were randomly selected for DNA sequencing, and the results were visualized by Chromas Lite program.

For PCR product direct sequencing, CsLBDP-5-P1 and CsLBDP-3-P2 were used to amplify the DNA fragments from genomic DNA. The PCR products were purified and subjected to direct sequencing using primer CsLOB1-P2 (5'-TGAGCAATGTGCACGTACTTTTGATACAGTGGTCTTC-3'). The results were analysed by Chromas Lite program.
Xcc-facilitated agroinfiltration in Duncan grapefruit

Duncan grapefruit (Citrus paradisi) was grown in a glasshouse at temperatures ranging from 25 to 30 °C. Before Xcc-facilitated agroinfiltration was carried out, the plants were pruned for uniform shoot establishment.

The detailed protocol for Xcc-facilitated agroinfiltration in citrus leaves was described previously (Jia and Wang, 2014), with minor modification. Briefly, Duncan leaves were inoculated with a culture of actively growing XccAgumC re-suspended in sterile tap water (5 × 10⁶ CFU/mL). Twenty-four hours later, the XccAgumC-treated leaf areas were agroinfiltrated with recombinant Agrobacterium cells harbouring GFP-p1380N-Cas9/sgRNA:cslob1 or p1380-ATHSP708P-GUSin (Jia and Wang, 2014). Four days after agroinfiltration, leaves were subjected to GFP observation or genomic DNA extraction.

GFP detection

Four days after Xcc-facilitated agroinfiltration with GFP-p1380N-Cas9/sgRNA:cslob1 or p1380-ATHSP708P-GUSin, GFP fluorescence in the treated leaves was visualized under illumination of an EBQ 100 isolated light source using a Zeiss Stemi SV11 dissecting microscope equipped with an Omax camera. The leaf was photographed using the Omax Touview software.

Agrobacterium-mediated Duncan grapefruit transformation

Citrus transformation was performed as reported before (Orbović and Grosso, 2015). In detail, about 2923 Duncan epicotyl explants were co-incubated with recombinant Agrobacterium cells harbouring binary vector GFP-p1380N-Cas9/sgRNA:cslob1. Five weeks later, about 839 shoots sprouted from these explants after co-inoculation. All explants were inspected for the presence of GFP fluorescence. In the initial screen, 15 shoots were designated as positive and micro-grafted on ‘Carrizo’ citrange rootstock plants [Citrus sinensis (L.) Osbeck × Poncirus trifoliata (L.) Raf.]. Out of these shoots, seven died upon grafting in vitro conditions before they were transferred to pots. Additional two plants were discarded based on unsatisfactory level of GFP fluorescence detected in their tissue during secondary inspection. The six remaining GFP-positive plants were used for further analysis.

The GFP-p1380N-Cas9/sgRNA:cslob1-transformed plants were subjected to PCR analysis with a pair of primers, 35SP-S-P1 (5’-ATCAAAAGGCCATGGAGTCAAA-3’) and NosP-3-P2 (5’-TTGTCGTTTCCCGCCTTCAGT-3’).

Next-generation sequencing analysis

Genomic DNA from six transgenic plants was used as template for PCR amplification using a pair of primers, CsLOB1-P1 (5’-TCTCATAACTACTACAACCCCAAGC-3’) and CsLOB1-P2 (Figure 1). All PCR products were pooled to construct the DNA library for sequencing using an Illumina HiSeq 2500 platform at Novogene (Beijing, China). For each sample, more than 50 000 paired-end reads were generated. After de-multiplex, barcode and primer deletion using custom Perl script, the raw reads were quality trimmed using sickle software with parameters average quality 30 and reads length threshold 200 bp (Fass et al., 2011). The remaining high-quality reads were clustered with a threshold of 100% pairwise identity using UCLUST (Edgar, 2010). The representative sequences from abundant clusters with relative abundance >1% were aligned using MEGA 6 (Tamura et al., 2013) and further analysed for mutation genotype.

Xcc infection assay

Wild-type Duncan grapefruit and CsLOB1-modified grapefruit lines were grown in a glasshouse. The same age leaves were inoculated with Xcc (5 × 10⁶ CFU/mL) using a needleless syringe. After inoculation, citrus canker formation was observed and photographed at different time points.

Analysis of potential off-targets

To analyse potential off-targets of GFP-p1380N-Cas9/sgRNA:cslob1 in CsLOB1-modified grapefruit lines, we analysed the putative off-targets using a web-based software (http://cbi.hzau.edu.cn/cgi-bin/CRISPR). Genomic DNA from CsLOB1-modified grapefruit lines was used as template, and the primers listed in Table S3 were used to amplify the fragment containing the off-targets. Finally, the PCR products were ligated with PCR-BluntII-TOPO vector for sequencing analysis.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Schematic diagram of GFP-p1380N-Cas9/sgRNA:cslob1. CaMV 35S and 35T, the cauliflower mosaic virus 35S promoter and its terminator; NosP and NosT, the nopaline synthase gene promoter and its terminator; LB and RB, the left and right borders of the T-DNA region; Flag-Cas9-NLS, the Cas9 endonuclease containing Flag tag at its N-terminal and nuclear location signal at its C-terminal; target, the 20 nucleotides of CsLOB1 highlighted by red, was conserved on both alleles; sgRNA scaffold, a synthetic single-guide RNA composed of a fusion of CRISPR RNA and trans-activating CRISPR RNA; NptII, neomycin phosphotransferase II; GFP, green fluorescent protein; CsVMV, the cassava vein mosaic virus promoter; PAM, protospacer-adjacent motif.

Figure S2. Representative chromatograms of CsLOB1 and its mutations in DLOB9 and DLOB10. Representative chromatograms of CsLOB1 and its mutations in DLOB9 transgenic plant (a, b) and #DLOB10 transgenic plant (c, d). The targeted sequence within CsLOB1 was shown by black lines, and the mutant site was pointed out by an arrow. Star indicates SNP.

Figure S3. The six Duncan transgenic lines showing differential resistance to Xcc. At 7 days postinoculation with Xcc (5 × 10^8 CFU/mL), severe canker symptoms were observed on wild type grapefruit, DLOB2 and DLOB3. Reduced canker symptoms were present on DLOB9, DLOB10, DLOB11 and DLOB12.

Figure S4. No visible phenotypic changes for GFP-p1380N-Cas9/sgRNA:cslob1-transformed Duncan grapefruit lines. The GFP-p1380N-Cas9/sgRNA:cslob1-transformed Duncan grapefruit lines were grown in glasshouse.

Table S1: Overview of the next generation sequencing data.

Table S2: Analysis of indel mutations for Type I CsLOB1 and Type II CsLOB1 in six transgenic Duncan grapefruit lines.

Table S3: Potential off-targets in transgenic Duncan grapefruit.