Engineering canker-resistant plants through CRISPR/ Cas9-targeted editing of the susceptibility gene CsLOB1 promoter in citrus

Presented By:
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Research Topics and Seminar (510)
30th Nov, 2021

Outline

- The selected paper
- Background
- CRISPR/cas9
- Paper objectives and abstract
- Methodology and Results
- Conclusion
- Possible applications

The selected paper

Plant Biotechnology Journal

Plant Biotechnology Journal (2017) **15**, pp. 1509–1519 doi: 10.1111/pbi.12733

Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene *CsLOB1* promoter in citrus

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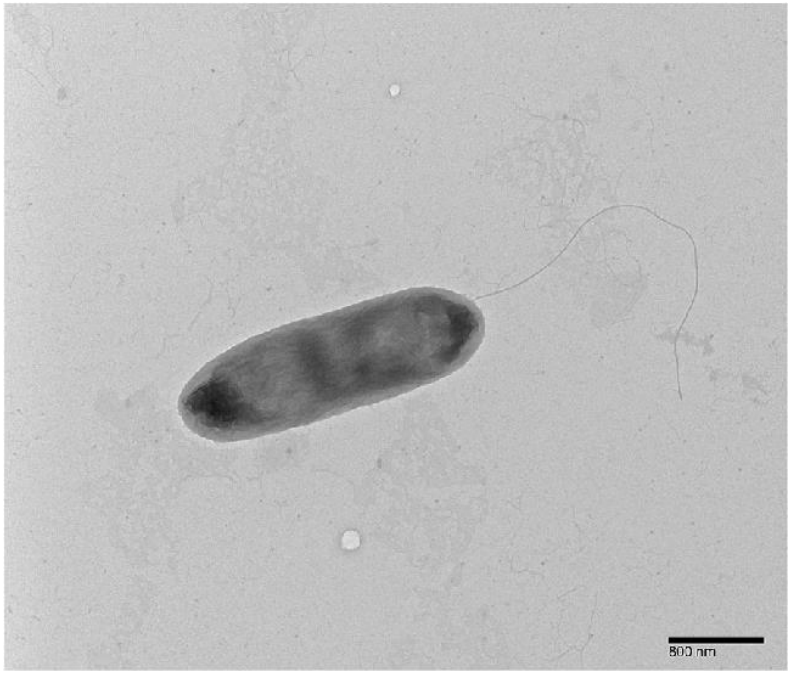
Paper Objective and Abstract

Summary

Citrus canker, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), is severely damaging to the global citrus industry. Targeted editing of host disease-susceptibility genes represents an interesting and potentially durable alternative in plant breeding for resistance. Here, we report improvement of citrus canker resistance through CRISPR/Cas9-targeted modification of the susceptibility gene *CsLOB1* promoter in citrus. Wanjincheng orange (*Citrus sinensis* Osbeck) harbours at least three copies of the $CsLOB1^{G}$ allele and one copy of the $CsLOB1^{-}$ allele. The promoter of both alleles contains the effector binding element (EBE_{PthA4}), which is recognized by the main effector PthA4 of *Xcc* to activate *CsLOB1* expression to promote citrus canker development. Five pCas9/CsLOB1sgRNA constructs were designed to modify the EBE_{PthA4} of the *CsLOB1* promoter in Wanjincheng orange. Among these constructs, mutation rates were 11.5%–64.7%. Homozygous mutants were generated directly from citrus explants. Sixteen lines that harboured EBE_{PthA4} modifications were identified from 38 mutant plants. Four mutation lines (S2-5, S2-6, S2-12 and S5-13), in which promoter editing disrupted *CsLOB1* induction in response to *Xcc* infection, showed enhanced resistance to citrus canker compared with the wild type. No canker symptoms were observed in the S2-6 and S5-13 lines. Promoter editing of $CsLOB1^{G}$ alone was sufficient to enhance citrus canker resistance in Wanjincheng orange. Deletion of the entire EBE_{PthA4} sequence from both *CsLOB1* alleles conferred a high degree of resistance to citrus canker. The results demonstrate that CRISPR/Cas9-mediated promoter editing of *CsLOB1* is an efficient strategy for generation of canker-resistant citrus cultivars.

What is citrus canker?

- **Citrus canker** caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) is one of the most destructive diseases causing severe yield losses in all citrus producing regions worldwide.



Transmission election micrograph of Xanthomonas citri

Strategies controlling citrus canker

- The primary strategy for control of citrus canker relies on an integrated disease control approach.

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Disadvantages of this approach:


high cost


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Disadvantages of this approach:

 high cost

 risks to human and animal health

 adverse environmental effects

- Breeding resistant cultivars is the most efficient and economical approach in the long term to control citrus canker.

Genetic engineering is the **FASTEST**

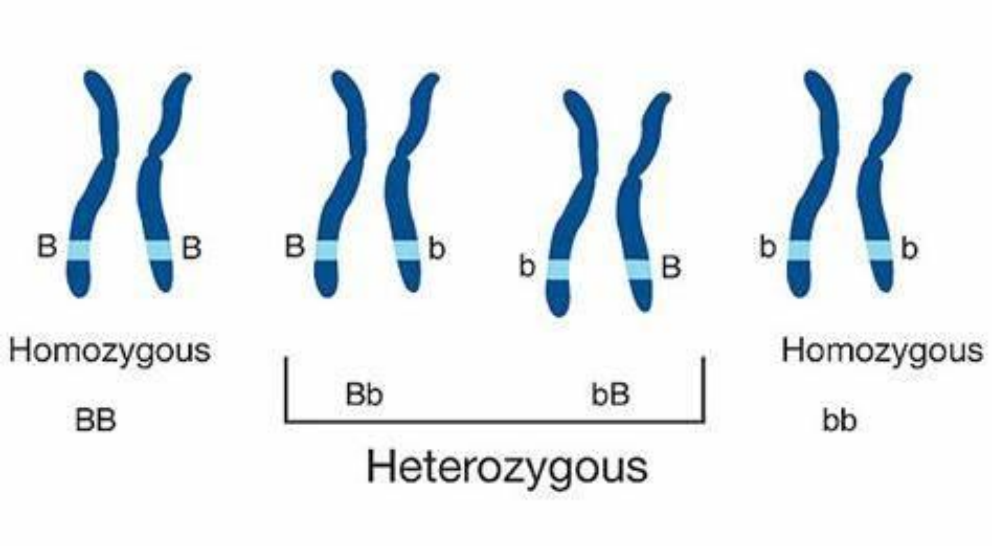
What is the problem?

- No active resistance genes have been identified in citrus

What is the problem?

- No active resistance genes have been identified in citrus

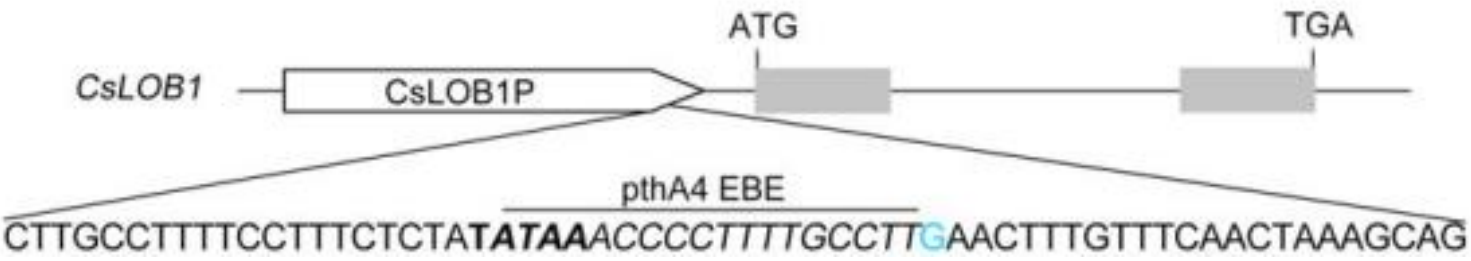
- High degree of heterozygosity
- Wide host range of Xcc



CsLOB1

- All plant genes that facilitate infection and support compatibility are considered to be **susceptibility genes**.

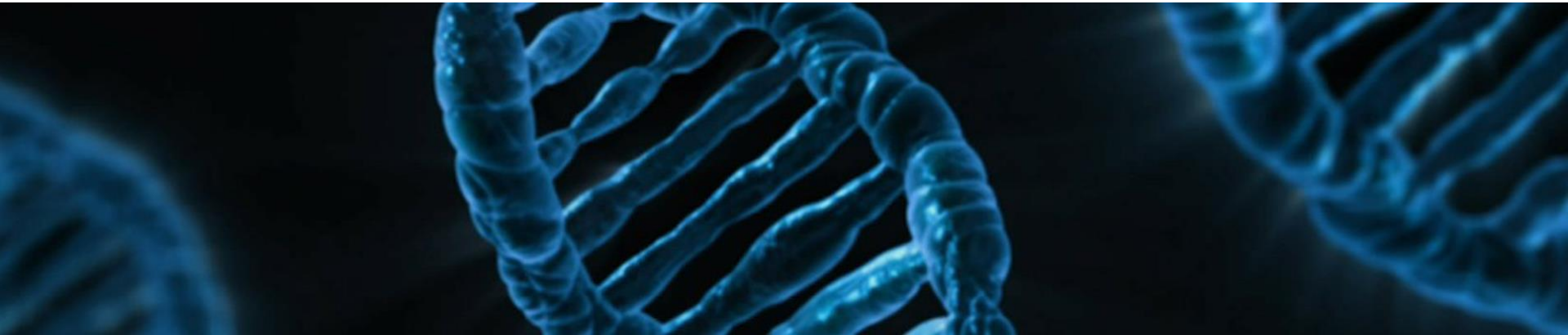
- **LATERAL ORGAN BOUNDARIES 1 (*CsLOB1*)** the susceptibility gene for citrus canker, plays a critical role in promoting pathogen growth and erumpent pustule formation



PthA4

- The main transcription activator-like (TAL) effector of Xcc, **PthA4**, specifically binds to EBE in the CsLOB1 promoter.

- Activate expression of CsLOB1 to favor citrus canker development.

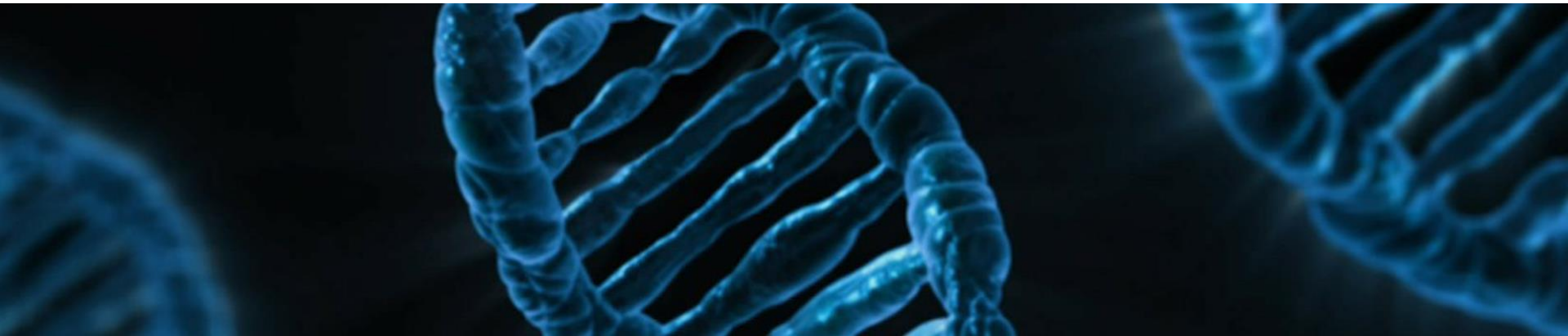


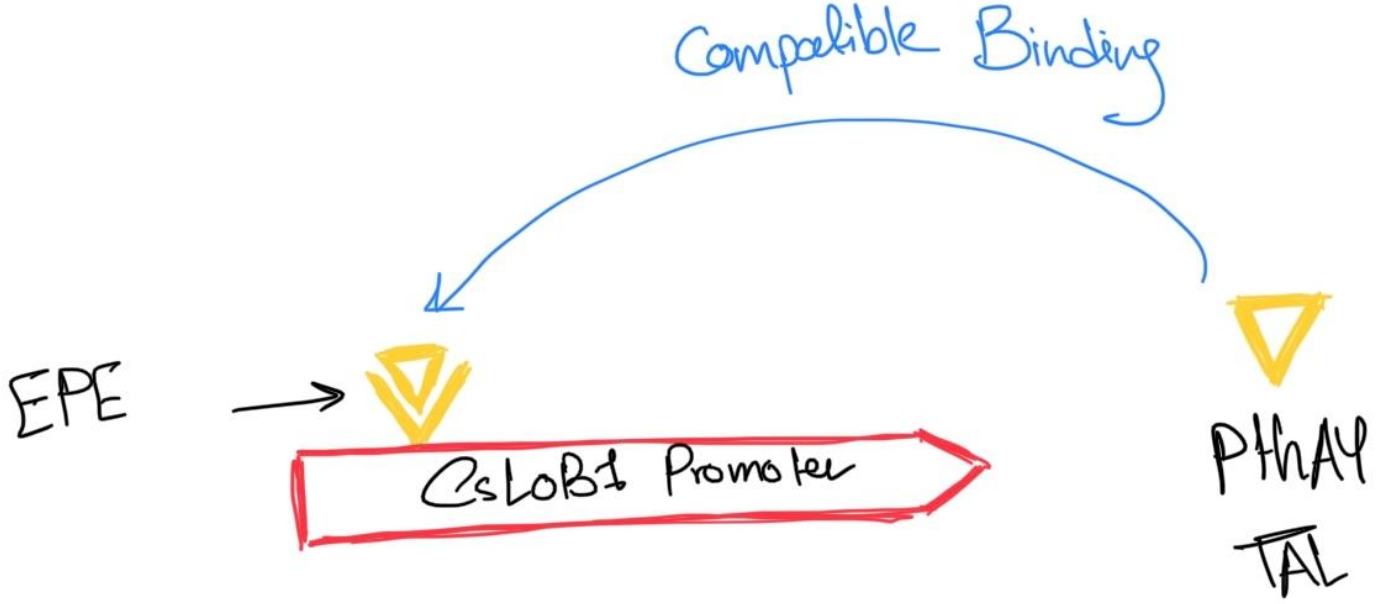
Solution

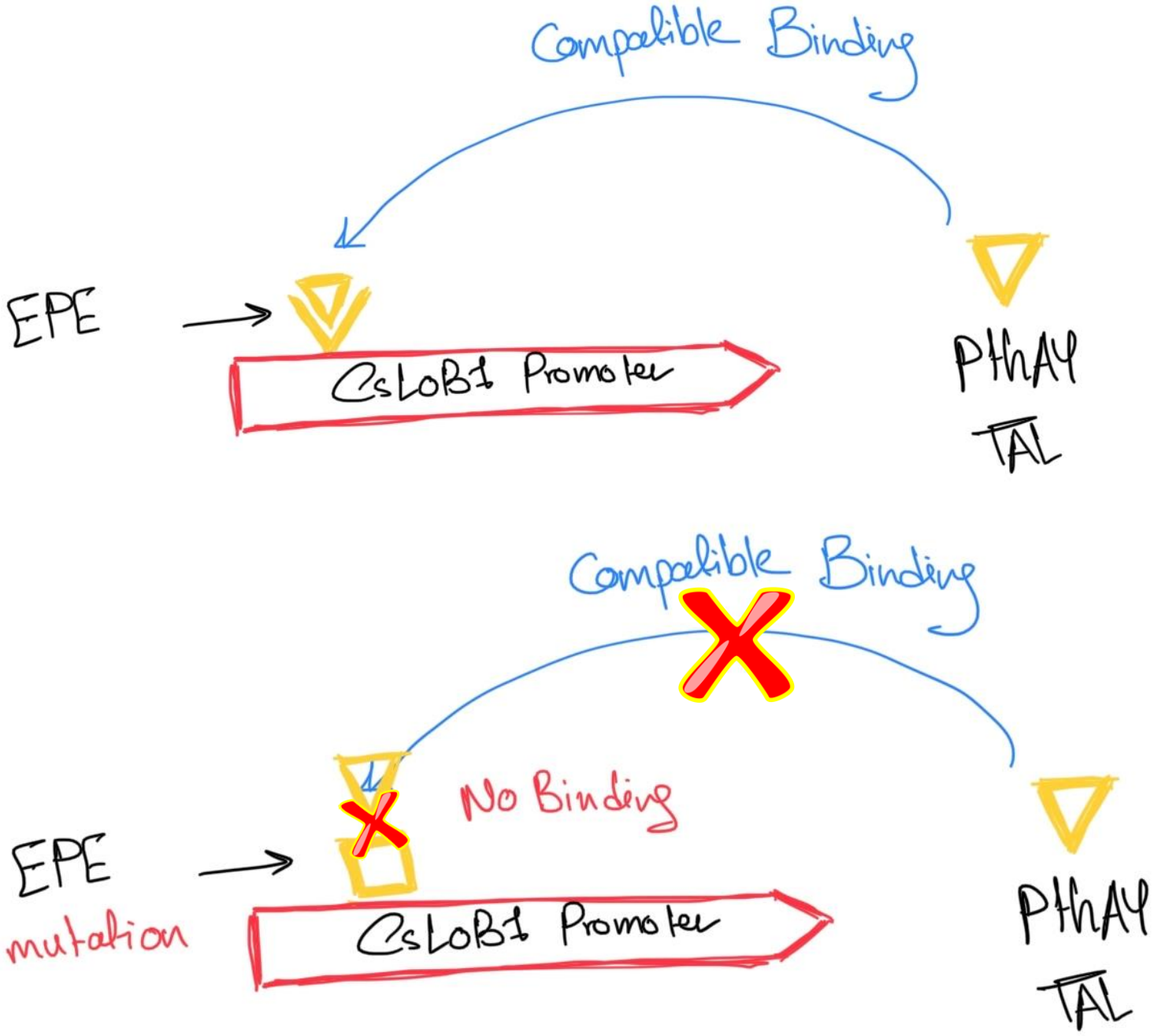
- **Mutation on EPE !**

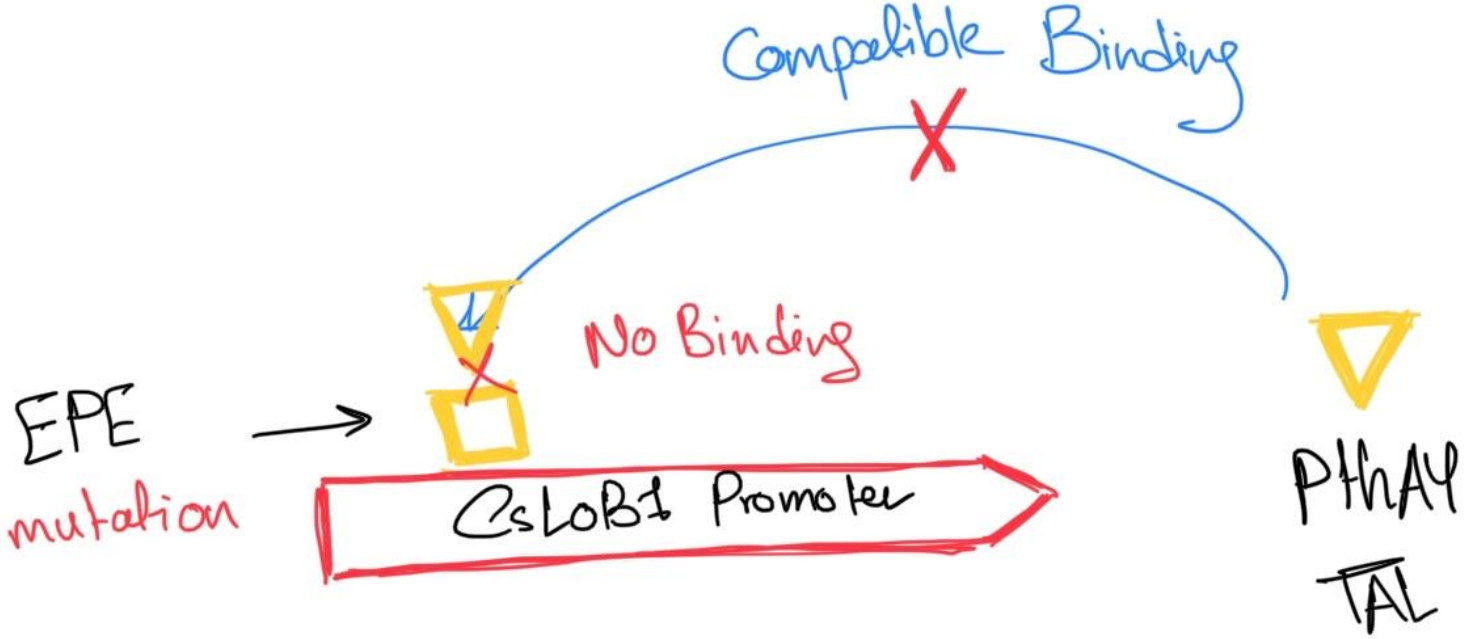
- Suppress expression of CsLOB1 gene, therefore no development of citrus canker!

- Such mutations do not interfere with the developmental functions of the targeted genes.

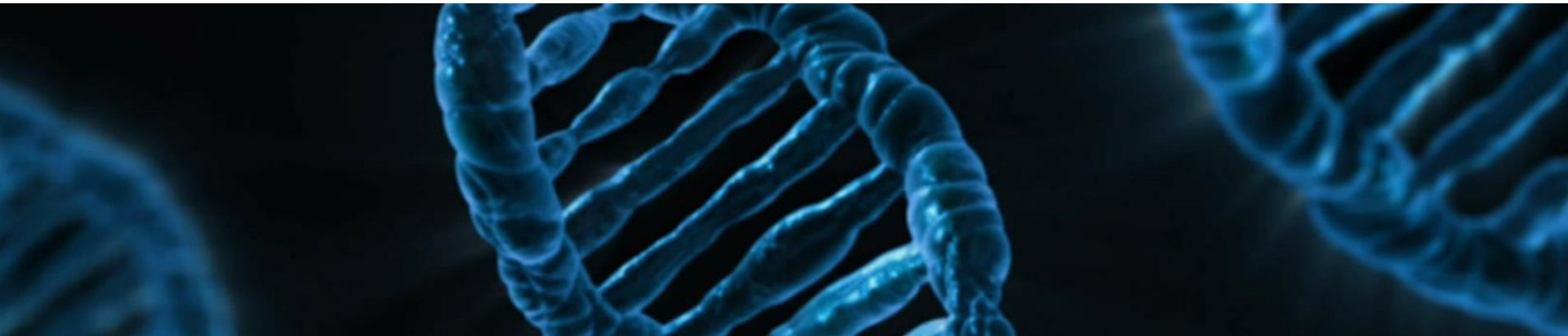



Compatible Binding
EPE
CsLOB1 Promoter
PthA4
TAL


Compatible Binding
EPE
CsLOB1 Promoter
PthA4
TAL
Compatible Binding
No Binding
EPE
mutation
CsLOB1 Promoter
PthA4
TAL

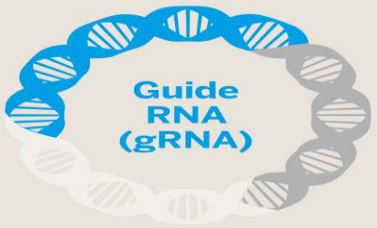


ONLY PthA4 EBE mutations that repress or abolish the TAL-inducible expression of CsLOB1 may enhance plant disease resistance.



CRISPR-Cas9

How the genome editor works



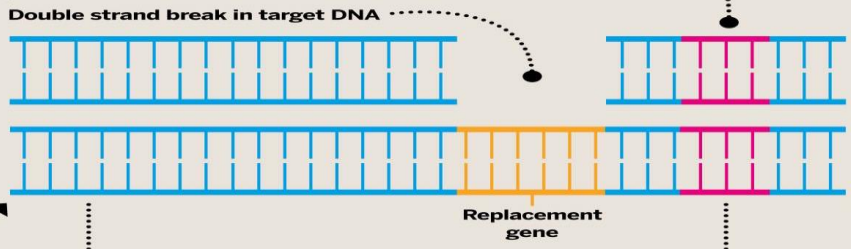
1 A cell is transfected with a DNA plasmid that expresses both the Cas9 protein and a sequence of guide RNA (gRNA), which matches that of the gene of interest.

2 Cas9 identifies the corresponding DNA sequence on the host cell's genome, and cuts both strands of DNA.

3a The cell's attempt to repair the break effectively **silences the targeted gene** by joining the cleaved DNA back together, using a process called non-homologous end joining (NHEJ).

OR

3b A **faulty gene can be 'corrected'** with a replacement segment of DNA, or **a new gene altogether can be introduced**. If a modified piece of DNA whose flanking regions match the target sequence is also supplied, then there is a good chance that it will recombine with the host DNA when the cut is made, thus introducing a new or replacement gene. This pathway is known as homology directed repair (HDR).



Cas9 requires a simple and common sequence of base pairs called a **PAM sequence** to actually bind to target DNA. This feature means bacteria can prevent Cas9 from chopping up important 'memorised' sequences of foreign DNA in their own genome – by ensuring there are no PAM sequences in those regions.

What next?

FOOD AND LIVESTOCK MODIFICATION
Researchers have already created plants and mammals with edited genomes. It is hoped such technology could help boost productivity and improve food security.

GENE DRIVE
Some genes are more likely to be passed on than others. If an 'edit' is linked to these genes, it will quickly spread through a wild population. That sounds alarming, but could help eradicate malaria-carrying mosquitos.

GENE THERAPY
Genetic disease could be treated by introducing gene editing systems into affected cells. Researchers in the USA are trialling this to treat HIV by knocking out the gene for the specific T-cell receptor that the virus targets.

HUMAN GERM LINE
Modifying human embryos, sperm or eggs would introduce changes to the genome of future generations. Some argue that other techniques, such as embryo screening, can just as effectively prevent genetic disease.

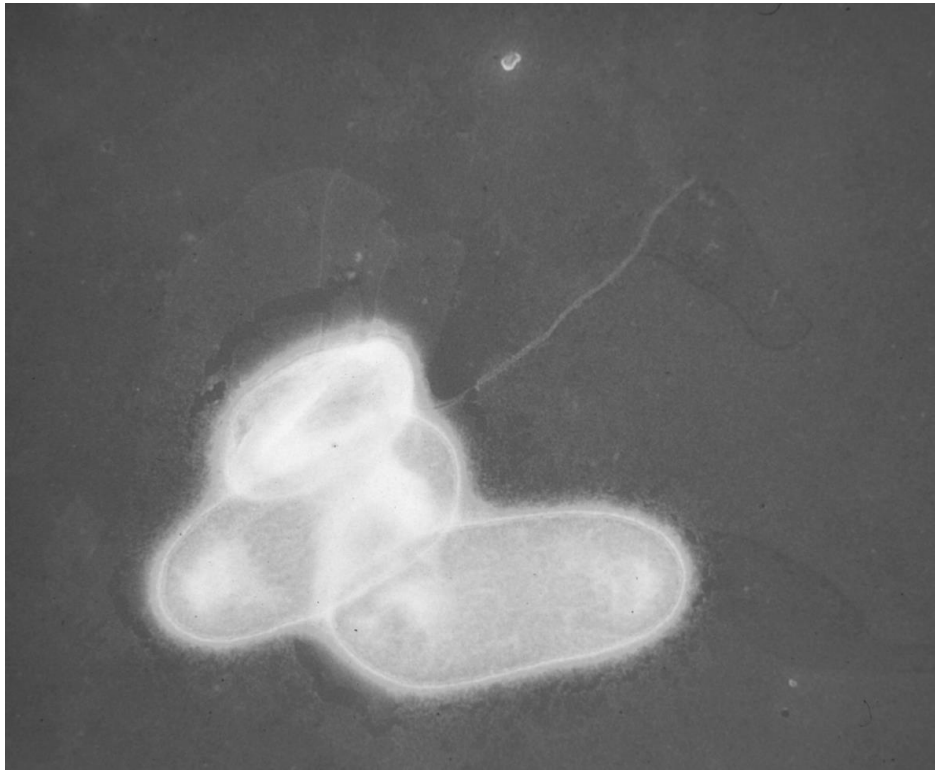
DESIGNER ORGANISMS AND MORE...
In future, could babies be 'designed' with a genome of our choosing? Could amateur biologists do their own gene editing outside regulatory systems?

Main sources

Wanjincheng orange (WJ)

***Xanthomonas citri* (*Xcc*)**

From infected sweet orange leaves

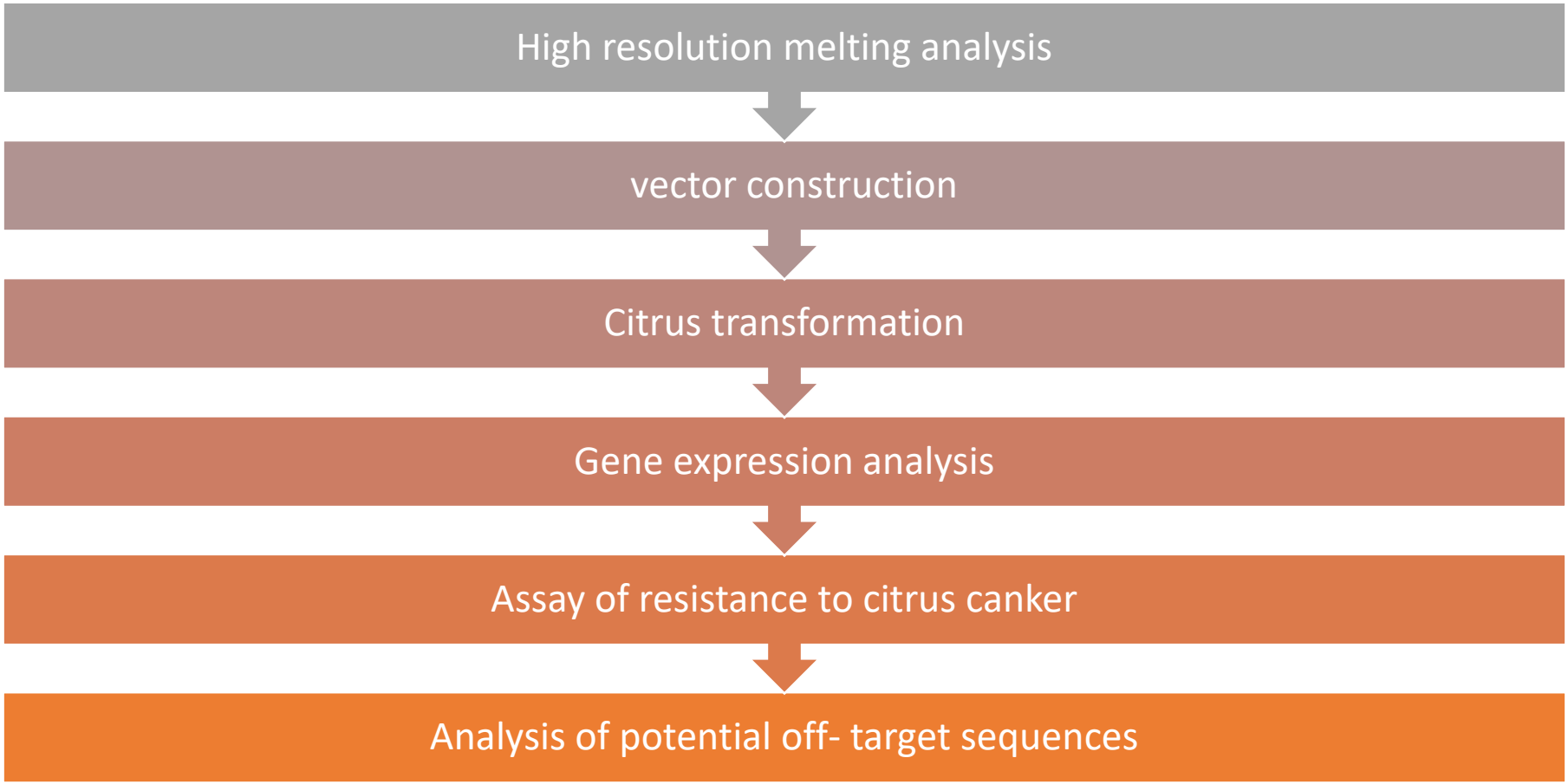


What are the Controls?

<u>Satsuma Mandarin</u> (SM)

<u>Chandler Pummelo</u> (CP)

Methodology Outline



Method and Results

- High resolution melting analysis (HRM)

- Extracted genomic DNA from citrus leaves
- Genotyping of the CsLOB1 promoter
- PCR
- Direct Sequencing of the PCR amplicons

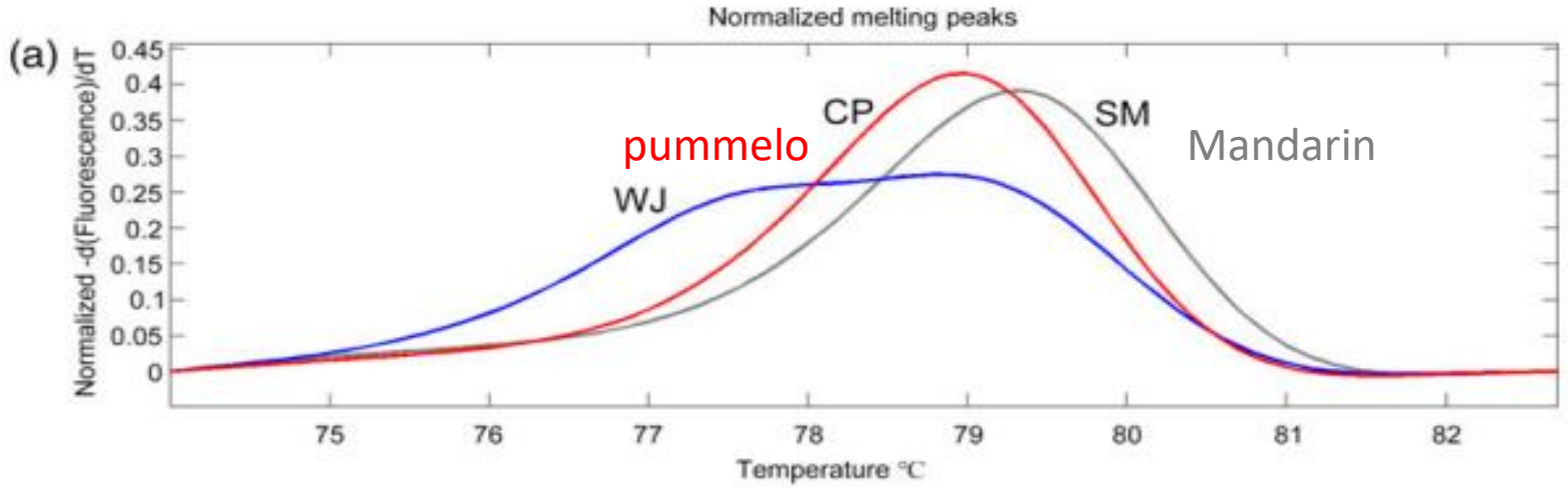


Figure1. a) High Resolution Melt (HRM) analysis is a powerful technique in molecular biology for the detection of mutations, polymorphisms and epigenetic differences in double-stranded DNA samples.

Three types of indel were present among the three species

Method and Results

- Direct Sequencing of the PCR amplicons

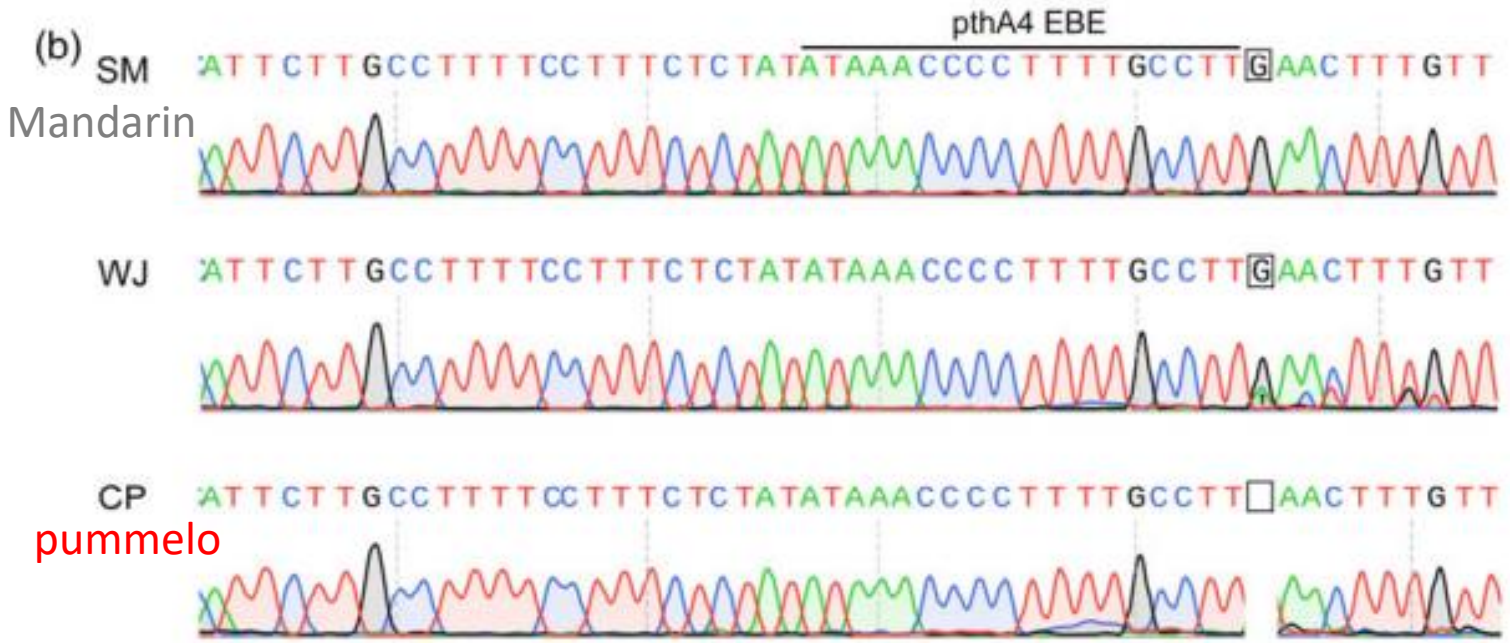


Figure.1 b) Direct sequencing analysis of the CsLOB1 promoter in Wanjincheng orange.

Method and Results

- Vector construction

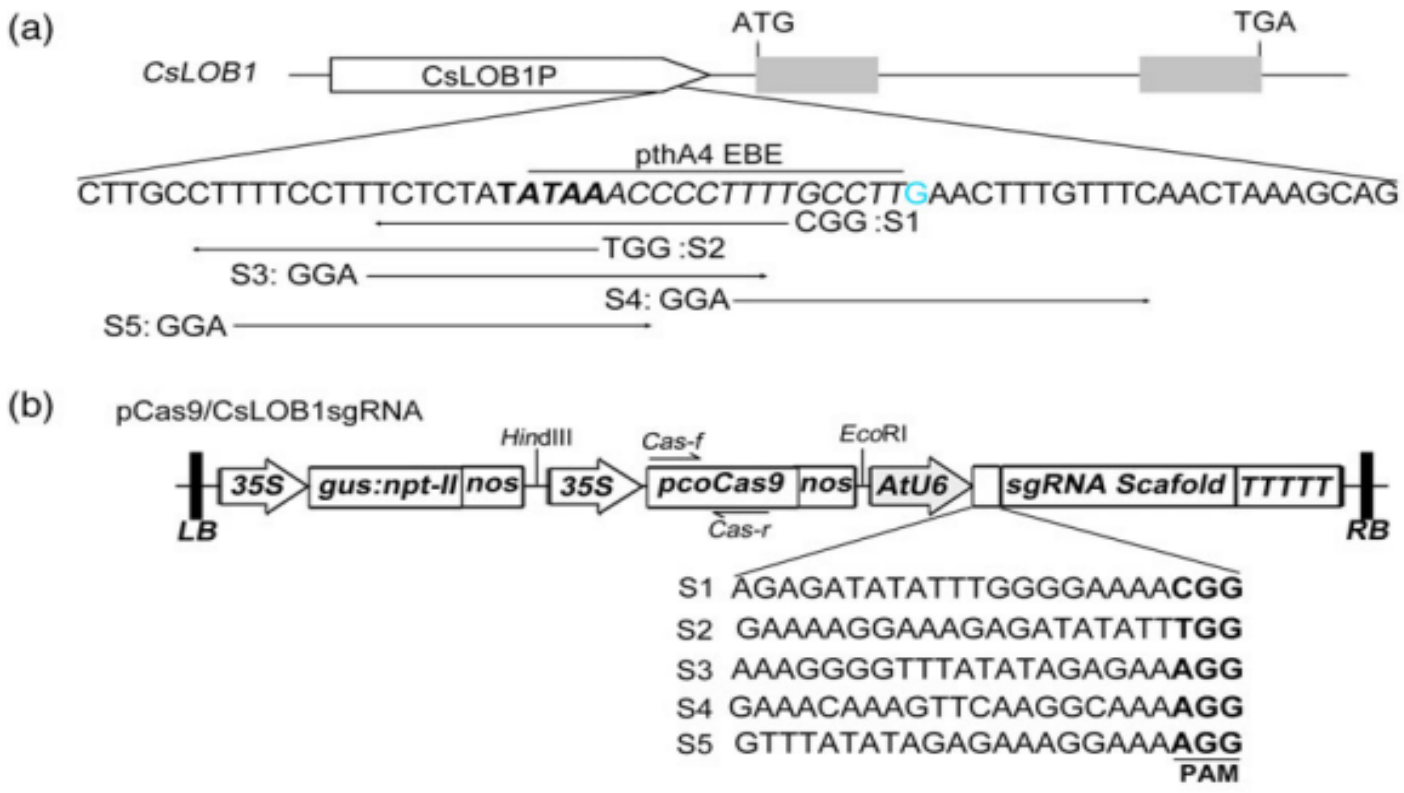
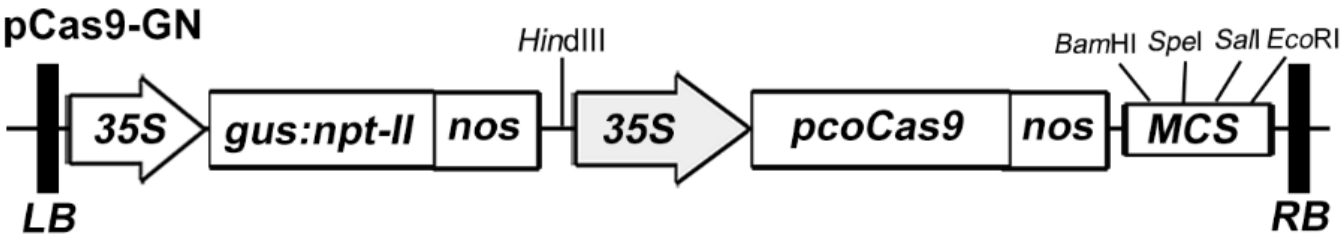


Figure 2 CRISPR/Cas9-mediated modification of the CsLOB1 promoter in Wanjincheng orange
(a) Schematic structure of CsLOB1
(b) Schematic diagram of pCas9/ CsLOB1sgRNA vectors

Method and Results

- Vector construction



The pCas9-GN vector (Figure S10) was used to construct CRISPR/ Cas9 expression vectors for citrus transformation

Method and Results

- Citrus transformation

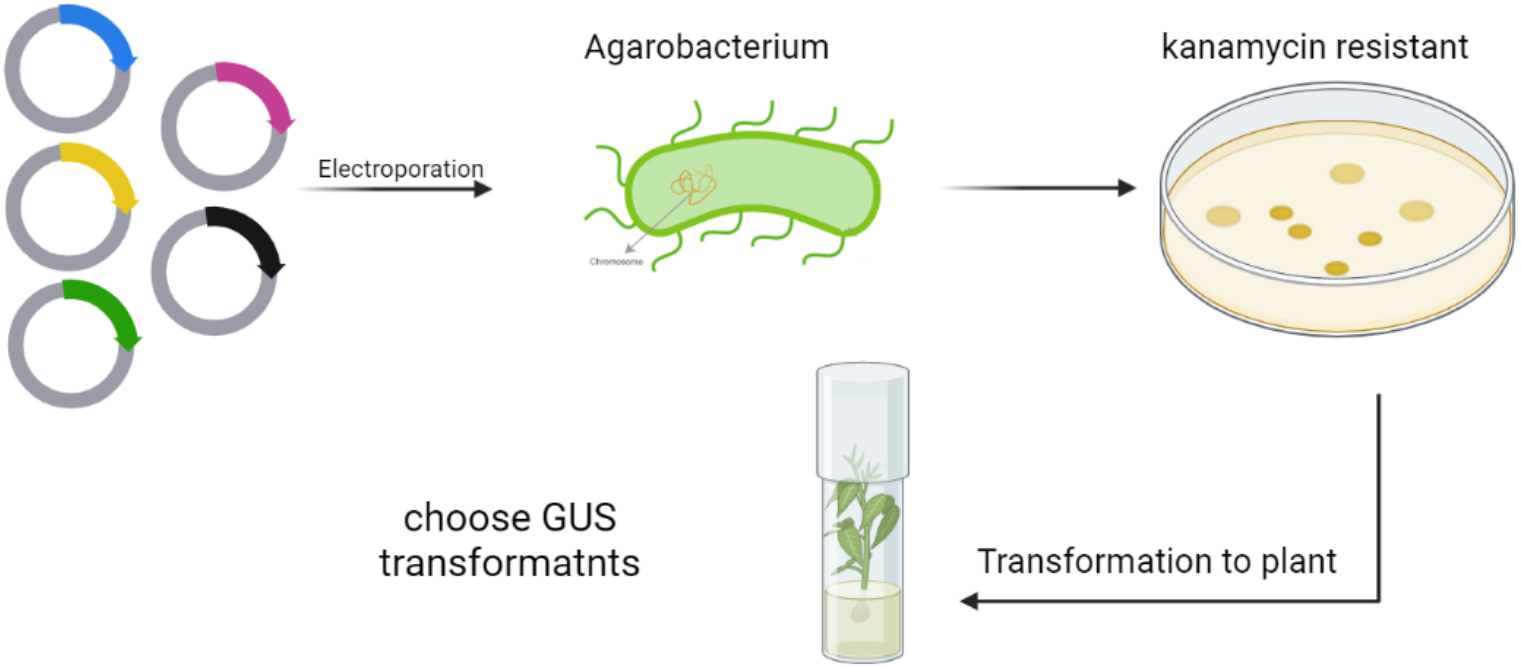


Figure created by Afnan Khaled

Method and Results

- Sequencing analysis for all transgenic plants

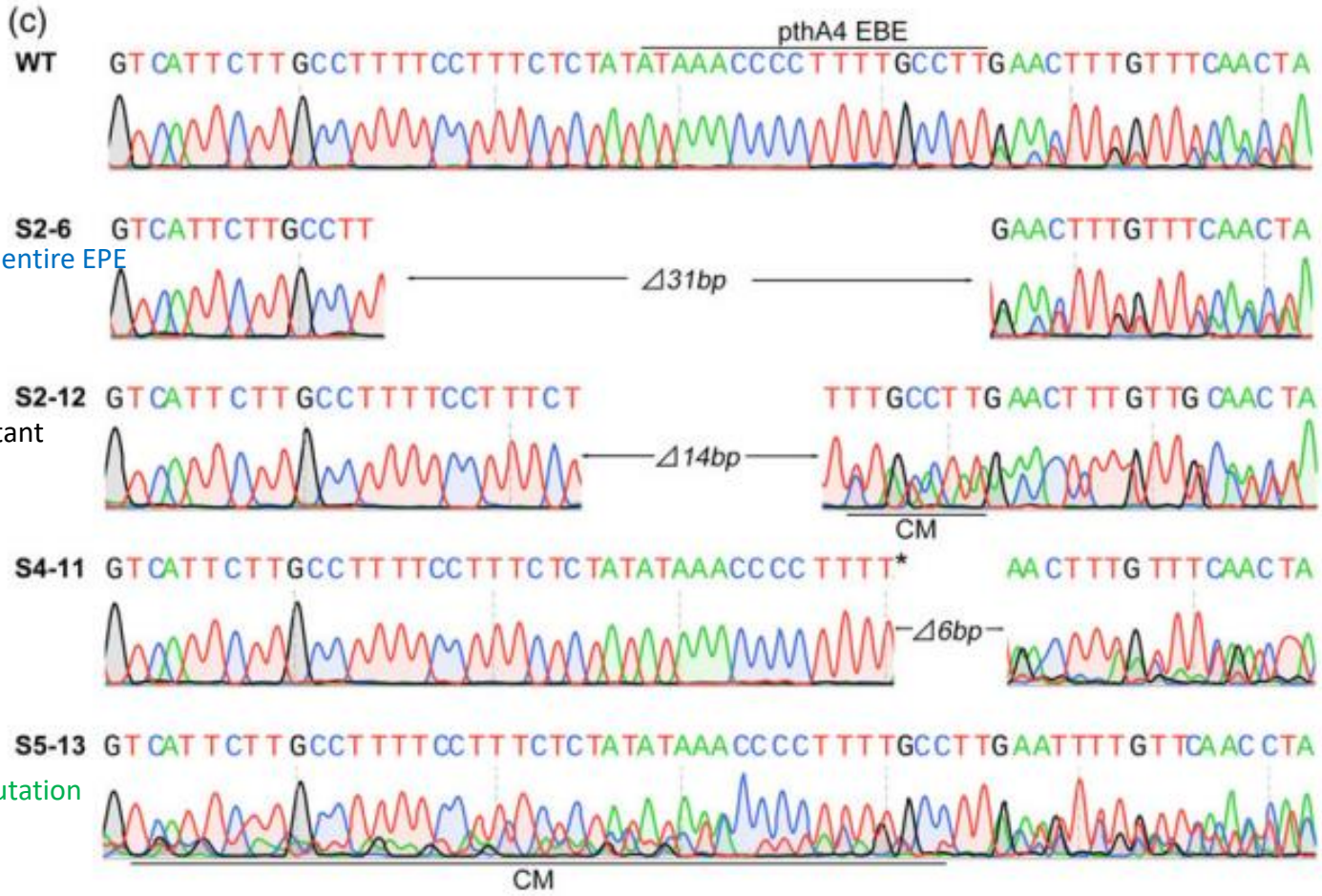


Figure.2 (c) Representative chromatograms of CsLOB1 promoter mutations. '⊿#bp' indicates the number of deleted nucleotides; '*' indicates an insertion; 'CM' indicates chimera mutations

Method and Results

- Gene expression analysis

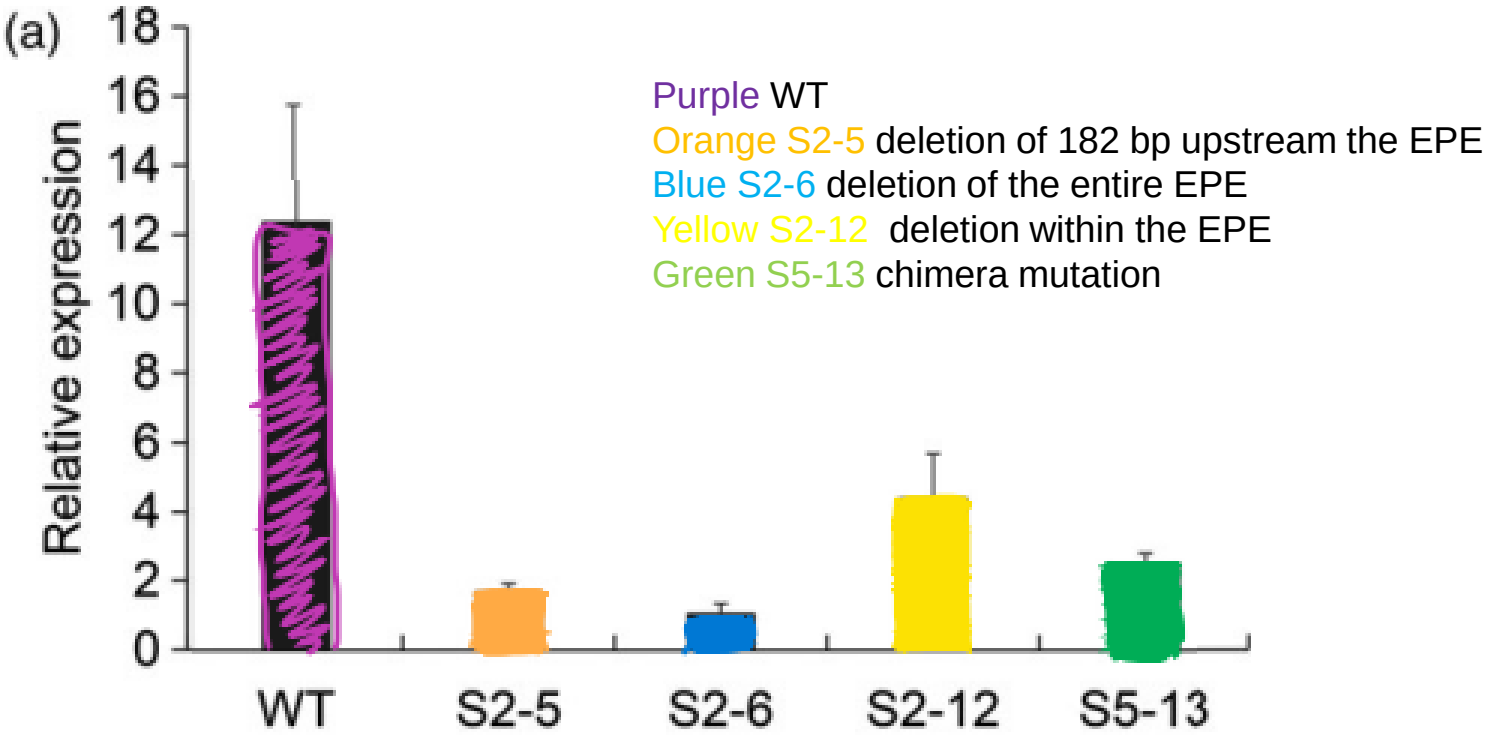
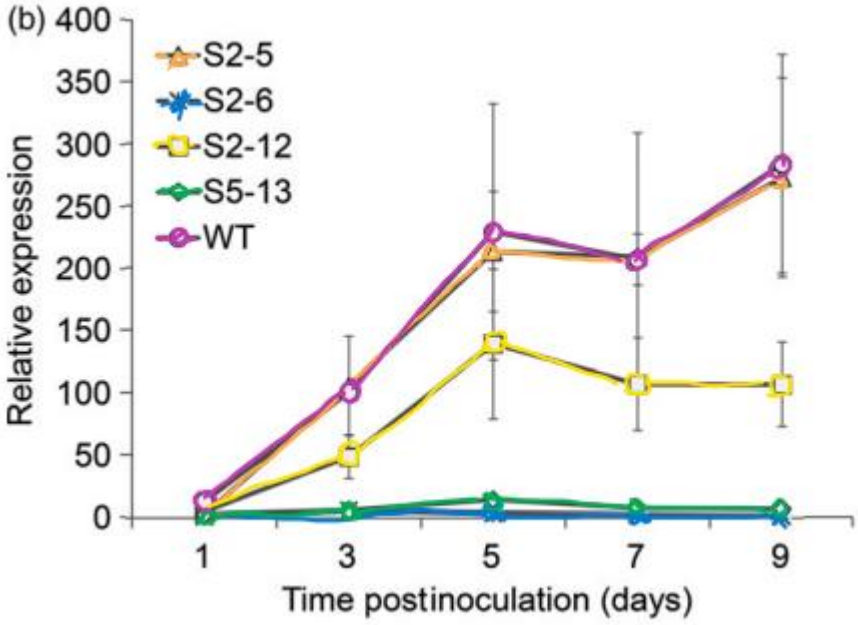


Figure.3 a) Expression of CsLOB1 in mutant plants after Xanthomonas citri subsp. citri (Xcc) inoculation. At 1 day postinoculation (dpi), CsLOB1 transcripts in leaves were analysed by quantitative real-time PCR (qPCR).

Method and Results

- Gene expression analysis



Purple WT
Orange S2-5 deletion of 182 bp upstream the EPE
Blue S2-6 deletion of the entire EPE
Yellow S2-12 deletion within the EPE
Green S5-13 chimera mutation

Figure.3 b) Time course of CsLOB1 expression in mutants after Xcc inoculation. Transcript levels of CsLOB1 in leaves were determined by qPCR

Method and Results

- Gene expression analysis

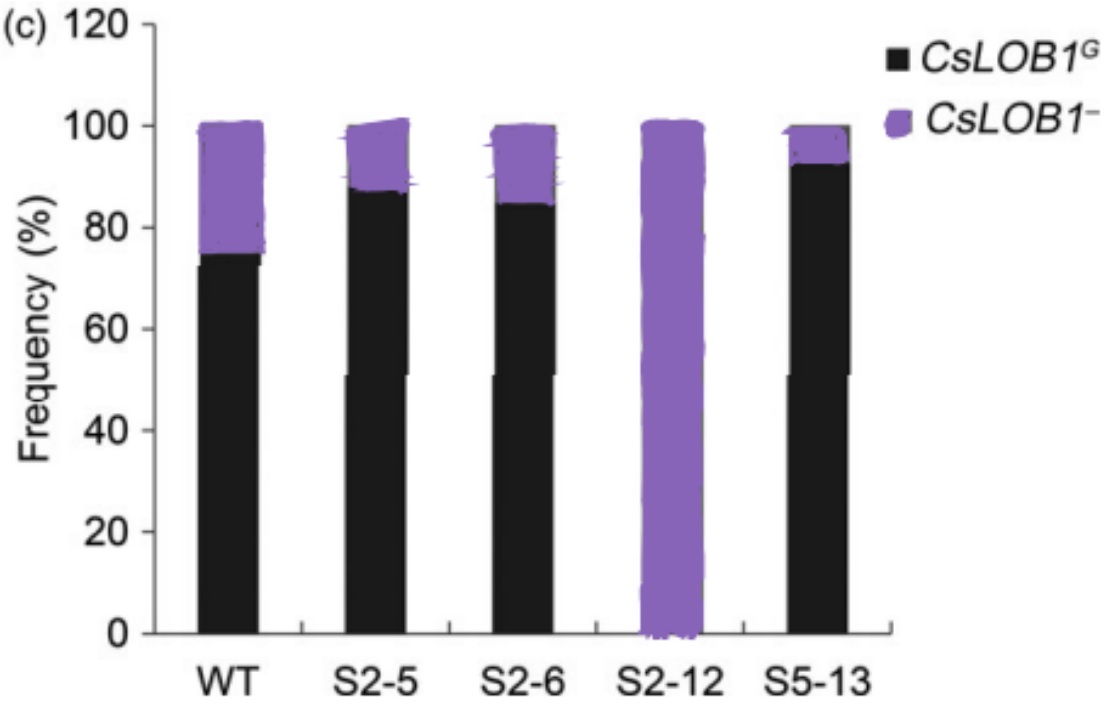


Figure.3 c) Statistical analysis of transcripts of CsLOB1G and CsLOB1- in citrus mutants. Frequency (%) indicates the percentage of each CsLOB1 mRNA out of the total mRNAs tested.

Method and Results

- Assay of resistance to citrus canker

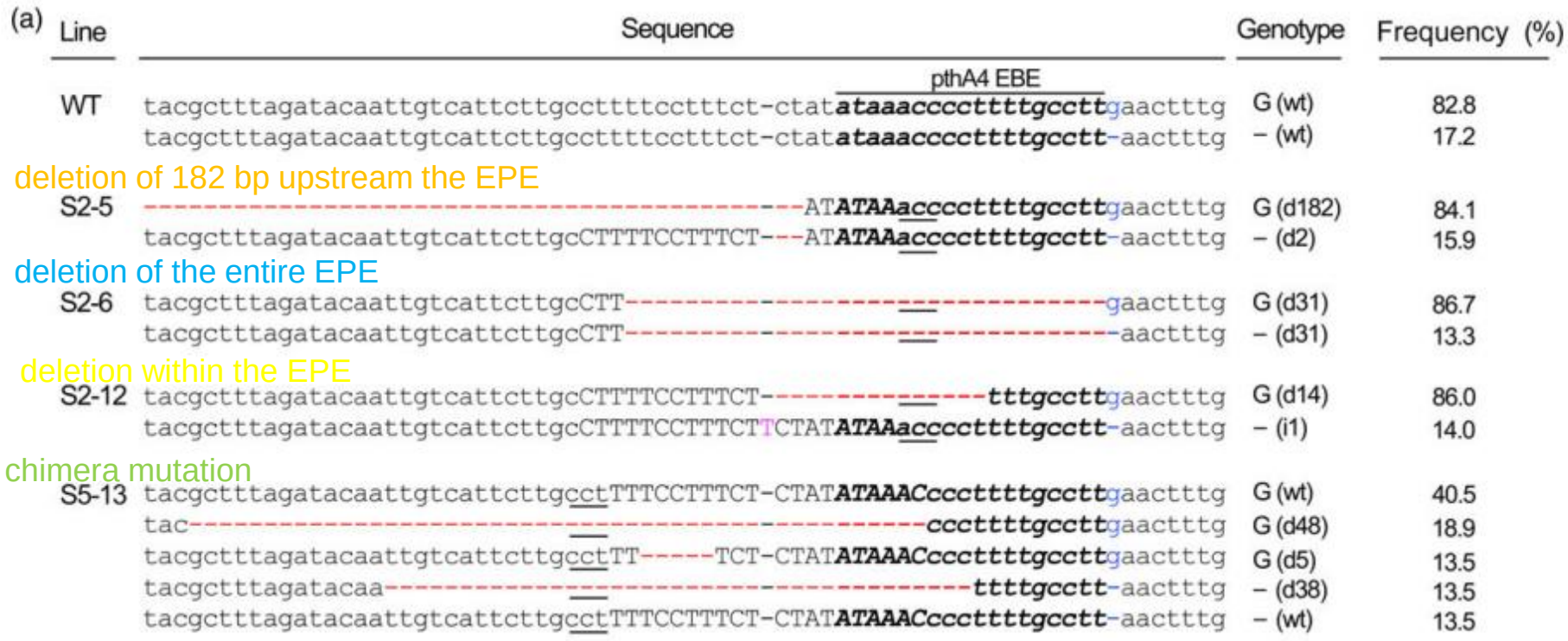


Figure 4 a) Representative sequences of CsLOB1 mutations induced by CRISPR/Cas9
Frequency (%) was calculated based on the number of clones with the same mutation out of the total number of clones sequenced.

Method and Results

- Assay of resistance to citrus canker

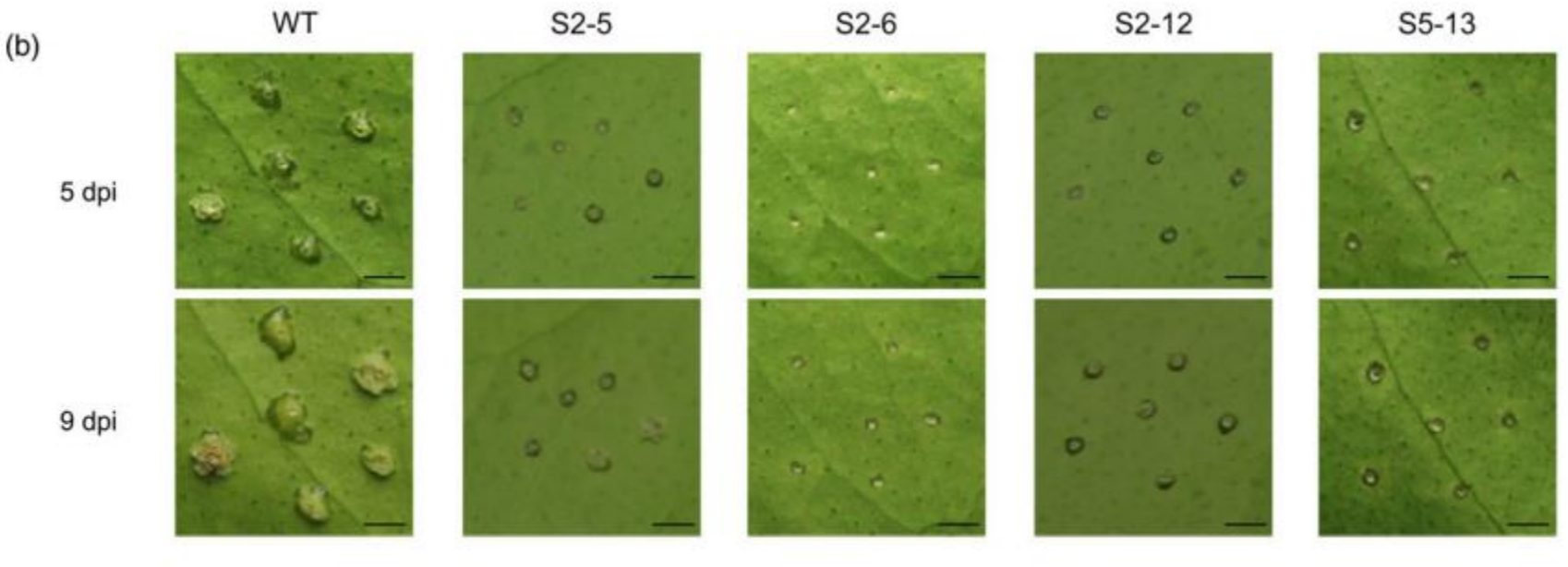


Figure.4 b) Assay of resistance to Xanthomonas citri subsp. citri (Xcc) in mutant plants

Purple WT
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Blue S2-6 deletion of the entire EPE
Yellow S2-12 deletion within the EPE
Green S5-13 chimera mutation

Method and Results

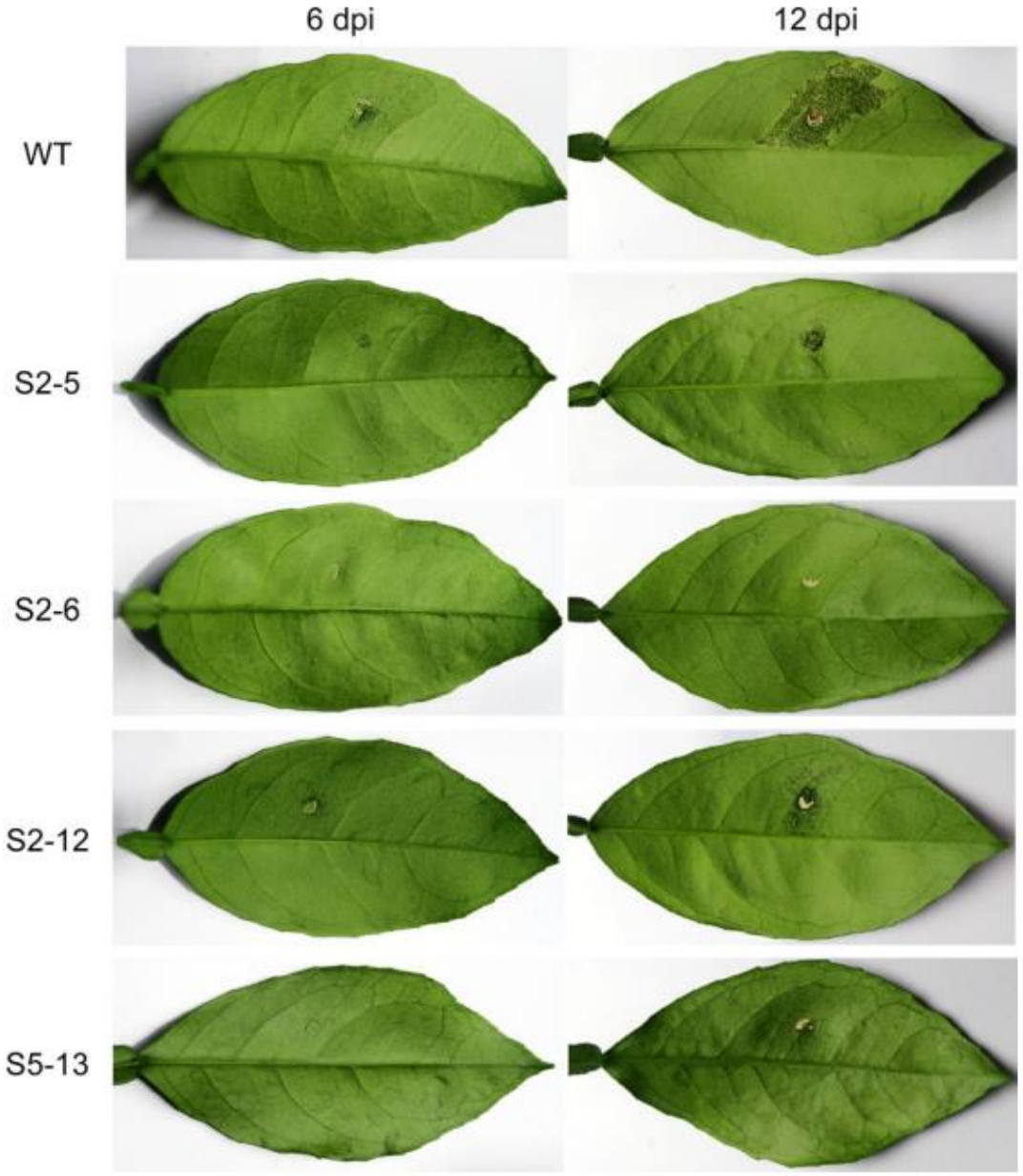

Figure 5 *In vivo* assay of citrus canker resistance in Wanjincheng orange (*Citrus sinensis* Osbeck) mutants. Leaves were infiltrated with *Xanthomonas citri* subsp. *citri* (*Xcc*) suspensions. At 6 days postinoculation (dpi), pustules were detected in wild type, but absent or significantly reduced in mutant plants. At 12 dpi, severe canker symptoms were detected in wild type, whereas markedly reduced symptoms were observed in S2-5 and S2-12. No canker symptoms were found in S2-6 and S5-13.

deletion of 182 bp upstream the EPE

deletion of the entire EPE

deletion within the EPE

chimera mutation

Method and Results

- Assay of resistance to citrus canker

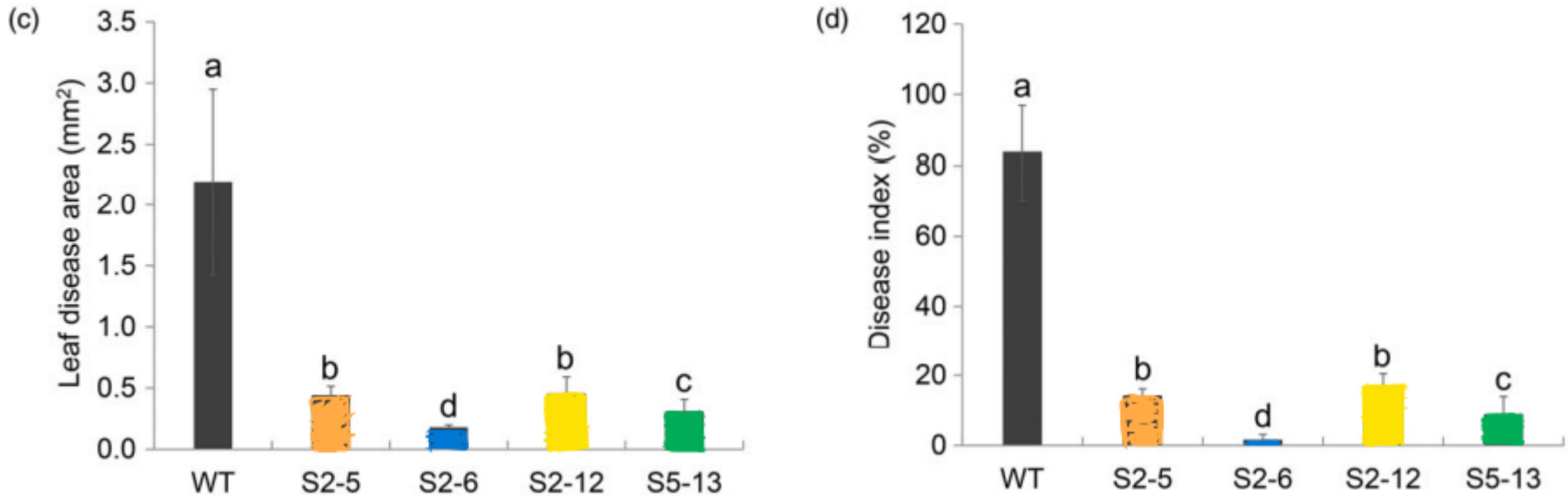

Figure.4 Disease lesion area (c) and disease index (d) of leaves of each mutation line were investigated at 9 dpi.

Method and Results

- Assay of resistance to citrus canker

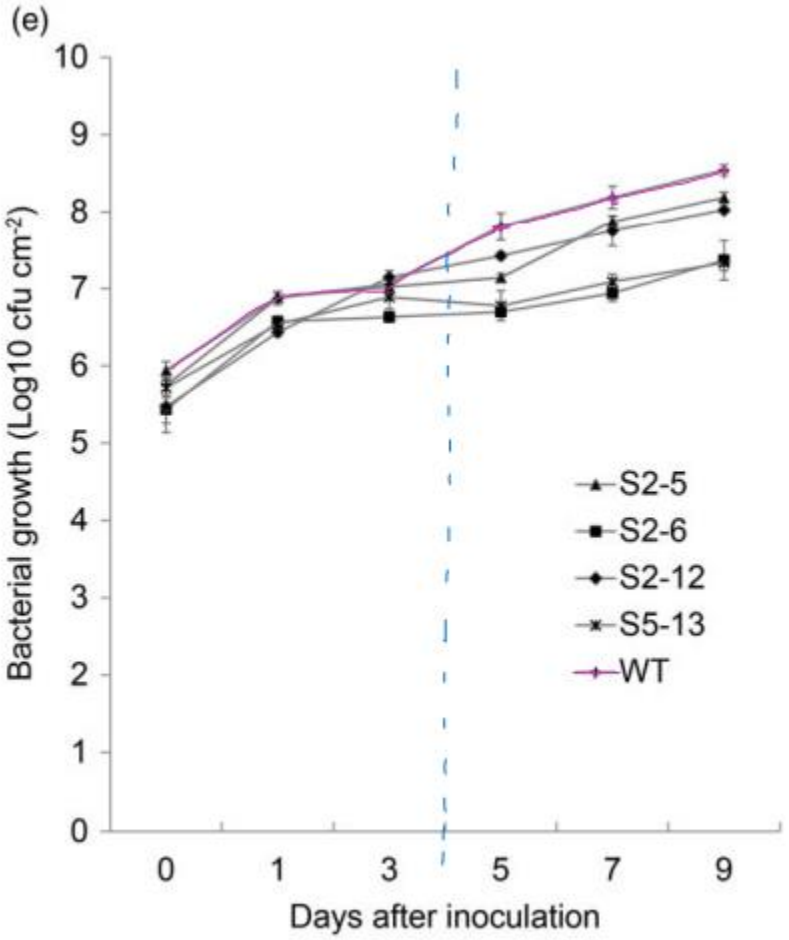

Figure.4 e) Growth of Xcc in leaves of mutant plants.

Method and Results

- Analysis of potential off-target sequences

- Mutations in all of the putative off-target loci were detected in the mutant lines tested. However, the off target frequencies were low (5.0–10.0%) and all of the mutations consisted of 1-bp point mutations

Conclusion

- Promoter editing of CsLOB1G alone was sufficient to enhance citrus canker resistance in Wanjincheng orange.

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- Promoter editing of CsLOB1G alone was sufficient to enhance citrus canker resistance in Wanjincheng orange.

- Deletion of the entire EBEPthA4 sequence from both CsLOB1 alleles conferred a high degree of resistance to citrus canker.

- The results demonstrate that CRISPR/Cas9-mediated promoter editing of CsLOB1 is an efficient strategy for generation of canker-resistant citrus cultivars.

- The present results show that CRISPR/Cas9- induced mutagenesis is precise and efficient in citrus, which will help to accelerate basic research and genetic improvement in citrus

Possible applications

- The concept can be applied to any plant containing susceptibility gene!
- Example: Powdery mildew (PM) caused by Podosphaera aphanisis a major fungal disease of cultivated strawberry
- Mildew resistance locus o (Mlo) genes in strawberries.


THANK YOU!