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REINALDO RODRIGUES DE SOUZA NETO

Characterization of *CsLIEXP1* as a new susceptibility gene to *Xanthomonas citri* subsp. *citri* and gene editing by CRISPR/Cas9 for citrus canker tolerance

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Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of PhD in Genetics and Molecular Biology on concentration area of Plant Genetics and Breeding.

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Orientador: ALESSANDRA ALVES DE SOUZA.

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Resumo

Cancro cítrico é uma doença causada por *Xanthomonas citri* subsp. *citri* (*Xcc*). A patogenicidade dessa bactéria é dependente da translocação de proteínas efetoras chamadas *Transcription Activator-Like Effectors* (TALEs) para dentro da célula hospedeira. Essas proteínas funcionam como fatores de transcrição nas plantas, promovendo a expressão de genes de susceptibilidade a doença. Em *Xcc*, o PthA4 é o principal TALE, essencial para regulação de genes do hospedeiro que vão facilitar a colonização da bactéria. Esse efetor transativa o gene *CsLOB1*, o qual é um fator de transcrição cuja expressão está associada ao desenvolvimento dos sintomas. Embora *CsLOB1* seja necessário para a indução de cancro em diferentes espécies de *Citrus*, outros genes podem também estar envolvidos no desenvolvimento do cancro. Desta forma, o objetivo desse estudo foi identificar novos genes de susceptibilidade de laranja doce a *Xcc* e usar a tecnologia CRISPR/Cas9 para edição e comprovação da funcionalidade do gene no desenvolvimento do cancro cítrico. Portanto, baseado em dados de transcriptoma, sete genes candidatos a serem regulados por PthA4 foram identificados e validados por expressão relativa. Entre eles, os genes *CsLOB1* e uma expansina mostraram os mais altos níveis de expressão em todas as variedades em resposta a infecção com *Xcc*, mas não para *Xcc*-Δ*pthA4*. Em ensaios de agroinfiltração, o gene expansina não foi regulado por PthA4, mas foi induzido por CsLOB1, o qual foi induzido por PthA4, indicando que o gene expansina é indiretamente regulada por PthA4. Portanto, esse gene foi renomeado para *CsLOB1-INDUCED EXPANSIN 1* (*CsLIEXP1*) Assim, o gene *CsLIEXP1* foi caracterizado como um gene de susceptibilidade indiretamente induzido por PthA4. Para comprovar seu papel no desenvolvimento do cancro cítrico, um vetor CRISPR/Cas9 foi desenvolvido com 3 sgRNA visando a edição do primeiro exon do gene *CsLIEXP1*. Vinte e duas linhagens transgênicas foram obtidas, e uma foi selecionada por *High Resolution Melting Analysis* (HRMA) como planta possivelmente editada, o que foi comprovado por sequenciamento genético. A linhagem H_CsLIEXP1_1 apresentou edição de 6,25% de cinco nucleotídeos e 37,5% de uma timina, sendo no total de 43,75% de edição. Esta planta foi desafiada com *Xcc* e afetou o crescimento bacteriano e desenvolvimento de sintomas. Dessa forma, foi demonstrado que a edição do gene *CsLIEXP1* interferiu no desenvolvimento dos sintomas de cancro cítrico e, consequentemente, afetou o crescimento bacteriana. Portanto, nesse estudo, um novo gene de susceptibilidade foi identificado e usando a abordagem de edição de genoma foi obtida planta mais tolerante ao cancro cítrico.

Abstract

Citrus canker is a disease caused by *Xanthomonas citri* subsp. *citri* (*Xcc*). The pathogenicity of this bacterium is dependent on the translocation of effector proteins called Transcription Activator-Like Effectors (TALEs) into the host cell. These proteins work as transcription factors in plants, promoting disease susceptibility genes expression. In *Xcc*, PthA4 is the main TALE essential for host gene regulation which will facilitate the bacterial colonization. This effector transactivates the *CsLOB1* gene, which one is a transcription factor, whose expression is associated with symptoms development. Although *CsLOB1* is required to induce canker on different species of *Citrus*, may other genes could be also involved in canker development. Thus, the goal of this study was to identify new susceptibility genes of sweet orange to *Xcc* and using CRISPR/Cas9 technology for editing and confirming of functionality in citrus canker development. Therefore, based on transcriptomic data, seven candidate genes to be regulated by PthA4 were identified and validated by relative expression. Among them, an expansin and Cs*LOB1* genes showed the highest expression levels in all varieties in response to *Xcc*, but not *Xcc*-Δ*pthA4* infection. In agroinfiltration assay, the expansin gene was not regulated by PthA4, but it was induced by CsLOB1, which one was induced by PthA4, thus, expansin is indirectly regulated by PthA4. Therefore, this gene was renamed for *CsLOB1-INDUCED EXPANSIN 1* (*CsLIEXP1*). So, the *CsLIEXP1* gene was characterized as a susceptibility gene indirectly induced by PthA4. To confirm its role in citrus canker development, a CRISPR-Cas9 vector was developed with 3 sgRNA targeting the editing of first exon of *CsLIEXP1* gene. Twnety-two transgenic lines were obtained, and one was selected by High Resolution Melting Analysis (HRMA) as putative edited plant, which was confirmed by genetic sequencing. The. H_CsLIEXP1_1 line presented editing of 6.25% of five nucleotides and 37.5% of one thymine, in total of 43.75% of editing. This plant was challenged with *Xcc* and the bacterial growth was affected as well as symptoms development. Thus, we demonstrated that editing of *CsLIEXP1* gene interfered in citrus canker development, and consequently, affected the bacterial growth. Therefore, in this study, a new susceptibility gene was identified and using genome editing approach citrus canker tolerant plant was obtained.

Summary

1. General introduction

1.1. Economic importance of sweet orange in Brazil

Sweet orange (*Citrus sinensis* (L.) Osbeck) is one of the most important products of Brazilian agrobusiness, once Brazil is the largest producer of this culture in the world (United States Department of Agriculture, 2022). The 2022/2023 harvest was estimated in approximately 317 million of sweet orange boxes (40.8 kg each one) in Brazilian citrus belt (<www.fundecitrus.com.br>). In addition, the production of sweet orange improves social development generating several direct and indirect jobs in different Brazilian states, such as São Paulo, Minas Gerais, Paraná, Sergipe, Rio Grande do Sul, Bahia, Rio de Janeiro and Goiás. São Paulo was the largest producer of sweet orange with approximately 77.5% Brazilian production in 2021 (www.ibge.gov.br/explica/producao-agropecuaria/laranja/br).

The majority sweet orange production is processed in Frozen Concentrated Orange Juice (FCOJ) and Not From Concentrated Orange Juice (NFC) (<www.citrusbr.com>). Brazil exports these products which is the largest exporter of sweet orange juice in the world (United States Department of Agriculture, 2022). In addition, *in natura* fruits are produced for internal consumption in Brazil, and other subproducts such as pectin, alcohol, D-limonene, essential oils and other derived from sweet orange demonstrating the economic importance of these culture (<www.citrusbr.com>; <www.citrosuco.com.br>).

Although the Brazil is the largest producer of sweet orange, some issues related with biotic and abiotic stresses have been affecting the productivity and the cost of production (Moreira et al., 2022). Among the biotic stresses, the bacterial diseases cause greater impact in sweet orange production, such as citrus variegated chlorosis (CVC) caused by *Xylella fastidiosa*, Huanglogbing (HLB) caused by *Candidatus* Liberibacter americanus and *Candidatus* Liberibacter asiaticus, and citrus canker caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) (Dalio et al., 2017).

1.2. Citrus canker

Citrus canker is a disease that affect all citrus varieties worldwide. However, different levels of citrus canker susceptibility are observed in all the citrus commercial varieties (De Carvalho et al., 2015). This disease is classified into three groups A, B and C depending on bacterium and host. The A group is caused *Xcc* and infects all commercial citrus species. The B group is caused by *Xanthomonas fuscans* subsp. *aurantifolii*, which infects some specific species such as Mexican lime and lemon, while the C group is caused by other strain of the

same bacteria, which causes disease only in Mexican lime and it is restricted to Brazil (Schaad et al., 2006; Al-Saadi et al., 2007; Cernadas et al., 2008).

Although three types of citrus canker were described, the symptoms are very similar varying only the host-pathogen specificity. The initial symptoms are water soaking, associated to hyperplasia and hypertrophy which induce pustule formation with yellow halo around (Figure 1) (Brunings and Gabriel, 2003). The development of symptoms promotes leaf fall (Brunings and Gabriel, 2003). The symptoms are the same in branches and fruits, but in fruits could induce premature fruit drop, which is associated with economic losses (Brunings and Gabriel, 2003; Gottwald et al., 2002).

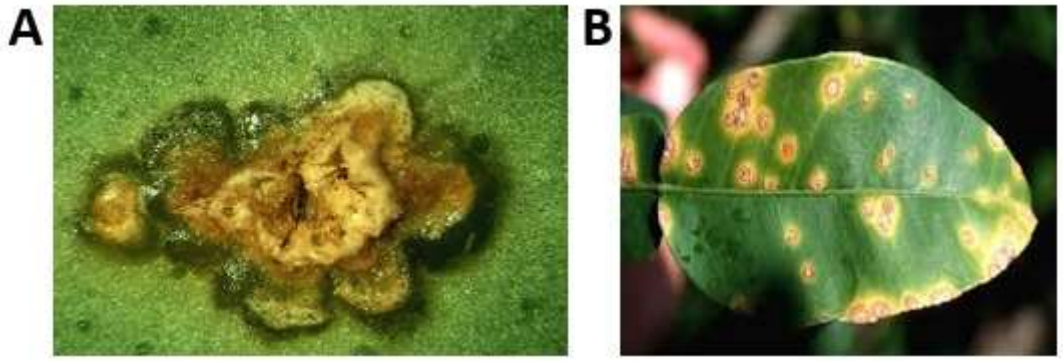


Figure 1. Citrus canker symptoms. A. Water soaking, hyperplasia, and hypertrophy symptoms. B. Citrus canker on leaf. Adapted from Gottwald et al., 2002.

Citrus canker control in Brazil is ruled by #21 federal normative instruction, which divides citrus production areas in four groups: area with absent pest; pest free area; area under eradication or suppression; and area under risk mitigation system <www.defesa.agricultura.sp.gov.br>. In cases of citrus canker detection, the infected plant is eradicated and all plants within a 30-meter radius around of eradicated plant are sprayed with a copper solution (0.01% copper) following #79 article from #21 federal normative instruction <www.defesa.agricultura.sp.gov.br>. The procedure describe in #79 article has been applied since 2013, which has been drastically increasing citrus canker incidence in São Paulo state (Behlau, 2021).

Xcc is naturally widespread to other plants by rain and wind, and on leaf, *Xcc* multiplies and forms a biofilm (Martins et al., 2020). The bacteria enter inside the leaf by natural openings such as stomate or by injuries (Brunings and Gabriel, 2003). Once inside the host, *Xcc* actives type III secretion system (T3SS) and injected effectors inside parenchymal cells in leaf mesophyll (Brunings and Gabriel, 2003). One effector class that is injected into plant cell is

Transcription Activator-Like Effector (TALE) (Brunings and Gabriel, 2003; Boch et al., 2009). These effectors active the transcription of host specific genes to improve colonization, which are associated with symptoms (Boch et al., 2009). These genes controlled by TALE are named as susceptibility genes (Boch et al., 2009).

1.3. Transcription Activator-Like Effectors

TALEs are proteins secreted by bacterial T3SS into plant cell, and responsible to trans-activate transcription of host specific genes which facilitate bacterial colonization (Boch and Bonas, 2010). TALEs are described with 3 domains: a central repeat domain, acid transcription activation domain (AD) and nuclear localization sites (NLS) (Figure 2A) (Boch et al., 2009). The AD domain is associated to induce the transcription, while NLS is responsible to drive TALE to plant cell nucleus (Boch et al., 2009). The central domain is compound by tandem repeats with 34 conserved amino acids in each repeat with high variability in 12^{th} and 13^{th} positions (Figure 2A) (Boch et al., 2009). This region is called by hypervariable region, and named as Repeat Variable Diresidue (RVD) (Boch et al., 2009; Scholze and Boch, 2010). TALES are differentiated from each other based on the number and array of RVDs (Boch et al., 2009). Each RVD binds with one nucleotide, and the RVD array is responsible to bind in Effector Binding Elements (EBE) region localized in promoter region, driving TALE to induce specific gene expression (Figure 2B) (Boch et al., 2009). In general, the first downstream nucleotide of EBE region is a thymine and based on RVD sequence, is predictable EBE region for each TALE (Boch et al., 2009). However, there are some RVDs that can bind to more than one nucleotide in lower frequencies (Figure 2C) (Boch et al., 2009), which suggests that TALEs could bind more EBEs.

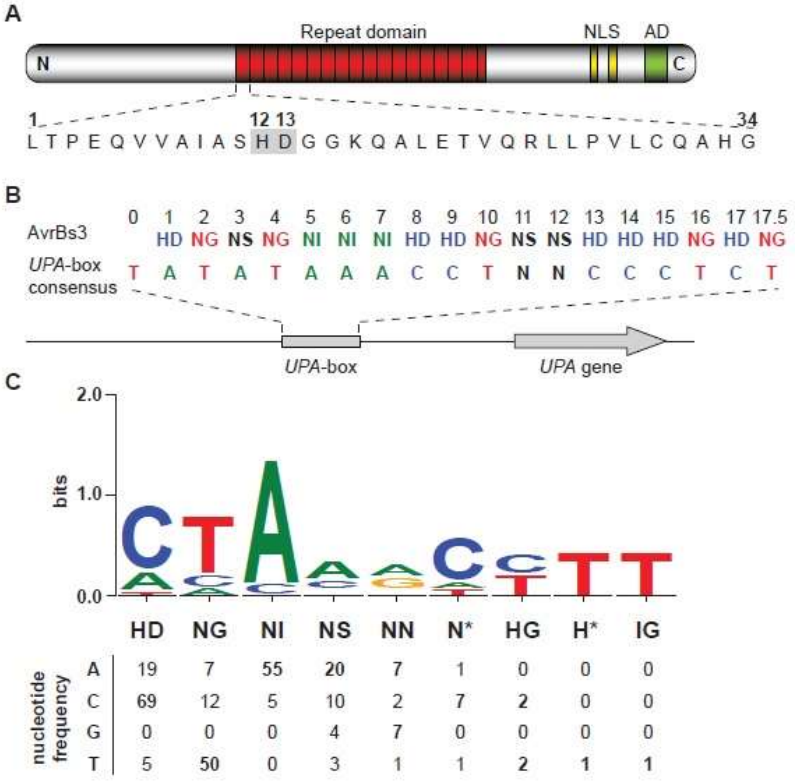


Figure 2. Model for TALE specificity. A. Structure of TALE. NLS is domains for nuclear localization site. AD is domain for acid transcriptional activation domain. Repeat domain is compound by repeats with 34 conserved amino acids each one. The 12th and 13th amino acid positions represent hypervariable region, which are responsible RVD name. B. EBE region with respective RVDs. C. Binding frequency of each RVD with different nucleotides. Source: Boch et al., 2009.

The *avrBs3* and *pth* genes are reported as TALEs genes (Boch and Bonas, 2010). In rice, the *PthXo1*, *PthXo6,* and *AvrXa7* TALE genes from *Xanthomonas oryzae* pv. *oryzae* induced, respectively, *Xa13*, *OsTFX1* e *Os11N3* expression (Römer et al., 2010). The EBE mutations suppressed gene expression, which shown those genes were modulated by respective TALE (Römer et al., 2010).

Xanthomonas gardneri mutant for *avrHah1* TALE was unable to promote water soaking, a characteristic symptom induced by this bacterium in tomato (Schwartz et al., 2017). Transcriptomic data revealed that AvrHah1 induced the expression of two transcription factors, and these genes associated to expression of a pectate lyase and a pectinesterase (Schwartz et al., 2017). The expression of pectate lyase was complemented using the artificial TALE strategy, which demonstrated the water soaking symptom was restored (Schwartz et al., 2017).

Four TALEs called PthA1-4 were reported for *Xcc* 306 strain, in citrus (da Silva et al., 2002). Although PthA1-3 have additive contribution for citrus canker symptoms, the PthA4 is the key virulence gene, once *pthA4*-mutant *Xcc* was unable to induce symptoms (Abe and Benedetti, 2016; Al-Saadi et al., 2007; Yan and Wang, 2011). Transcriptomic data demonstrated several genes were up-regulated in presence of PthA4, including genes associated to cell proliferation and cell wall remodeling (Hu et al., 2014; Pereira et al., 2014). One of the highest induced genes was the *LATERAL ORGAN BOUNDARIES 1* (*CsLOB1*), which one is a transcription factor associated to induce cell wall remodeling and cell proliferation (Duan et al., 2018; Zou et al., 2021). The *CsLOB1* was reported to be PthA4 target and a citrus canker susceptibility gene (Hu et al., 2014; And and Benedetti, 2016; Duan et al., 2018; Zou et al., 2021).

As mentioned above, TALEs induce plant gene expression, and they are essential key virulence factor for bacteria from *Xanthomonas* genus colonizes its hosts. The identification of these susceptibility genes controlled by TALES are extremely important to develop strategies aiming plant disease resistance. In this approach, CRISPR/Cas is a promise and revolutionary technology for gene editing which can disrupt the susceptibility gene or even EBE region, generating resistant plants.

1.4. CRISPR/Cas for gene editing in plants

For many years, studies were focused on to identify genes associated to plant disease resistance (van Wersch et al., 2020). The introduction of these genes in other varieties by plant breeding or transfer these genes for other species by genetic transformation was widely used aiming pathogen resistance (Fonseca et al., 2019). However, in plant-pathogen interaction studies, it was reported pathogens using effectors to regulate plant susceptibility gene expression, as described above (Boch et al., 2009). Nowadays, these susceptibility genes have great importance with the advances in genetic engineering, once those genes could be targets for knockout by genome editing aiming obtain new disease resistance varieties.

Clustered Regularly Interspaced Short Palindromic Repeats associated with the endonuclease Cas9 (CRISPR/Cas9) is a powerful technology used in plant genome editing (Jinek et al., 2012). The system work using a CRISPR RNA (crRNA), which is a single strand RNA with 20 nucleotides responsible to anneal with DNA target (Jinek et al., 2012). For Cas9 recruiting, it is essential a sequence upstream of target DNA named protospacer adjacent motif (PAM) (Jinek et al., 2012). PAM sequence could variate depending on Cas9 source, but the most used is from *Streptococcus pyogenes*, which PAM sequence is NGG (any nucleotide

followed by two guanines) (Pickar-Oliver and Gersbach, 2019). Other element is the trans-activating crRNA (tracrRNA), which hybridizes 5' portion with crRNA 3' portion by base pairing (Jinek et al., 2012). The trackRNA is necessary to form a threedimentional structure with crRNA and recruits Cas9 (Jinek et al., 2012). As a biotechnological application, the crRNA and trackRNA were fused in a single guide RNA (sgRNA) to drive Cas9 for target sequences (Figure 3A) (Jinek et al., 2012).

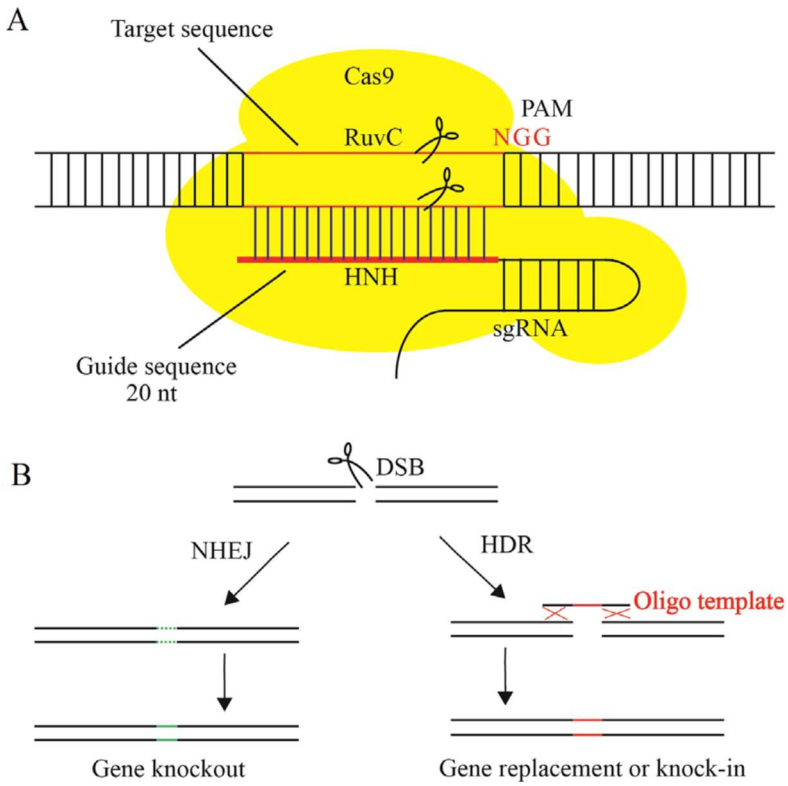


Figure 3. Schema of gene editing using CRISPR/Cas9. A. sgRNA driving Cas9 for genome editing. PAM is protospacer adjacent motif. sgRNA is single guide RNA. RuvC and HNH are two endonuclease domains. B. DNA repair mechanisms. DBS is double strand break. NHEJ is non-homologous end joining. HDR is homology directed repair. Source: Liu et al., 2017.

The efficiency of this technology is due to the specificity of sgRNA to drive Cas9 and easy handling of this system. In addition, Cas9 has two endonuclease domains, which are RuvC and HNH responsible to induce double strand break (DSB) in the same position (Figure 3) (Jinek et al., 2012). The DSB can be repaired in two ways, which are homology directed repair (HDR) and non-homologous end joining (NHEJ) (Liu et al., 2017). In HDR, a third element it is necessary, a double strand DNA with homology at the ends to induce homology recombination with target sequence (Figure 3B) (Liu et al., 2017). In NHEJ, the repair

mechanism induces indels in target sequences, which could generate frameshift and gene knockout (Figure 3B) (Liu et al., 2017).

In last years, genome editing technology has been advancing and Cas9 enzymes from other bacteria besides *S. pyogenes* has been used (Pickar-Oliver and Gersbach, 2019). In addition, the Cas12 enzyme could be used as alternative for genome editing (Schindele et al., 2018). Cas12 has some differences from Cas9, such as sgRNA three-dimensional structure, PAM sequence, DBS position and edited sequence (Schindele et al., 2018). Other genome editing technologies are DNA base editor and prime editor, which use modified Cas9, such as dead and nickase Cas9 (dCas9 and nCas9) in association with other proteins to generate small changes in DNA, for instance nucleotide substitution or small indels (Kantor et al., 2020). Because these new technologies, the system has been called by CRISPR/Cas.

Many plant species already been edited, for example tobacco, soybean, *Arabidopsis thaliana*, citrus, potato, apple, maize, banana, sorghum, rice, watermelon and others (Jia and Wang, 2014; Jiang et al., 2013; Kaur et al., 2018; Li et al., 2015; Liang et al., 2014; Nerkar et al., 2022; Nishitani et al., 2016; Wang et al., 2015; Wang et al., 2021). In citrus, *CsLOB1* was described as citrus canker susceptibility gene regulated by a TALE from *Xcc*, named PthA4 (Hu et al., 2014). *CsLOB1* gene was edited in exon and promoter regions, and edited plants were more resistant to citrus canker (Jia et al., 2017; Jia et al., 2022b; Jia et al., 2022a; Jia and Wang, 2020; Peng et al., 2017). In rice, the *OsSWEET11*, *OsSWEET13* and *OsSWEET14* genes are susceptibility genes modulated by different TALEs from *Xanthomonas oryzae* (Verdier et al., 2012). These genes were edited in promoter and exon regions, which generated resistance to *Xanthomonas oryzae* (Oliva et al., 2019; Xu et al., 2019). Thus, genome editing of S genes, modulated by TALEs, is a promise strategy to obtain plant disease resistance.

2. Hypothesis and Goals

Other genes, in addition to *CsLOB1*, are associated with susceptibility of sweet orange to *Xanthomonas citri* subsp. *citri*, and editing these genes generates citrus canker tolerant plants.

To prove this hypothesis the following objectives were developed:

1. Prospect and validate new susceptibility genes for citrus canker disease using genomic analysis and functional biology.

2. Editing the susceptibility gene found in sweet orange by CRISPR/Cas9 and evaluate the possible tolerance to citrus canker disease.

3. Chapter 1: The expansin gene *CsLIEXP1* is a direct target of CsLOB1 in citrus

3.1. Abstract

Transcription Activator-Like Effectors are key virulence factors of *Xanthomonas*. They are secreted into host plant cells and mimic transcription factors inducing expression of host susceptibility (S) genes. In citrus, *CsLOB1* is the direct target of PthA4, the primary effector associated with citrus canker symptoms. *CsLOB1* is a transcription factor, and its expression is required for canker symptoms induced by *Xanthomonas citri* subsp. *citri*. Several genes are up-regulated by PthA4, however, only *CsLOB1* was described as an S gene induced by PthA4. Here, we investigated whether other up-regulated genes could be direct targets of PthA4 or CsLOB1. Seven up-regulated genes by PthA4 were investigated, however an expansin-coding gene was more induced than *CsLOB1*. In *Nicotiana benthamiana* transient expression experiments, we demonstrate that the expansin-coding gene, referred here to as *CsLOB1-INDUCED EXPANSIN 1* (*CsLIEXP1*), is not a direct target of PthA4, but CsLOB1. Interestingly, *CsLIEXP1* was induced by CsLOB1 even without the predicated CsLOB1 binding site, which suggested that CsLOB1 has other unknown binding sites. We also investigated the minimum promoter regulated by CsLOB1, and this region and LOB1 domain were conserved among citrus species and relatives, which suggests that the interaction PthA4-*CsLOB1-CsLIEXP1* is conserved in citrus species and relatives. This is the first study that experimentally demonstrated a CsLOB1 downstream target and lay the foundation to identify other new targets. In addition, we demonstrated that the *CsLIEXP1* is a putative S gene indirectly induced by PthA4, which may serve as the target for genome editing to generate citrus canker resistant varieties.

3.2. Introduction

Transcription Activator-Like Effector (TALE) is a class of effectors mostly present in *Xanthomonas* genus (Boch and Bonas, 2010; Ference et al., 2018). TALEs function as key virulence factors in many plant pathogens that infect rice, cassava, tomato, pepper, cotton, wheat, and citrus (Charkhabi et al., 2017; Cox et al., 2017; Hu et al., 2014; Marois et al., 2007; Verdier et al., 2012; Zárate-Chaves et al., 2021). TALEs are injected into the cytosol of host cells by the bacterial Type III Secretion System (T3SS), and subsequently translocated into the nucleus via their nuclear localization signal (NLS) (Boch and Bonas, 2010). In the nucleus, the central repeat domain of TALEs binds to an effector binding element (EBE) region which is

found within promoters of host target genes to drive their expression (Boch et al., 2009). The central domain is composed of tandem repeats of 33-34 amino acids, which are very conserved with the exception of the 12th and 13th amino acids (Boch and Bonas, 2010). These two amino acids, called Repeat Variable Diresidue (RVD), facilitate the binding of a specific nucleotide within the EBE (Boch et al., 2009). The combination between number of repeats and the type of RVD define the binding affinity to the EBE region (Boch and Bonas, 2010). Genes which are transcriptionally induced by TALEs through direct interaction with the EBE region are called "susceptibility genes", because their induction is associated with symptom development and bacterial growth.

Xanthomonas citri subsp. *citri* (*Xcc*) is the causal agent of citrus canker disease, which causes significant economic losses in many citrus growing regions worldwide. Most commercial citrus varieties are susceptible to *Xcc* with few exceptions such as Kumquat (de Carvalho et al., 2015; Duan et al., 2022). The initial citrus canker symptoms are hyperplasia and hypertrophy followed by water soaking, which promotes pustule formation surrounded by yellow halo (Brunings and Gabriel, 2003). Four TALEs, called PthA1-4, were identified in the genome of the *Xcc* model strain 306 (da Silva et al., 2002). Although *pthA1*, *pthA2* and *pthA3* contribute to canker symptom development, *pthA4* is the primary pathogenicity factor of *Xcc*, and deletion of the *pthA4* gene (*Xcc*-Δ*pthA4*) eliminates the ability of *Xcc* to induce citrus canker symptoms (Abe and Benedetti, 2016; Al-Saadi et al., 2007; Yan and Wang, 2011). Transcriptomic analysis indicated that several genes related to cell wall remodeling and cell proliferation were upregulated by PthA4, for instance pectate lyases, endoglucanase, cyclins, expansins and transcription factors (TFs) (Hu et al., 2014; Pereira et al., 2014).

LATERAL ORGAN BOUNDARIES 1 (*LOB1*) encodes a transcription factor (TF) which is highly induced by PathA4 and belongs to the LOB domain (LBD) family, a TF class associated with expression of cell wall remodeling and cell proliferation genes (Hu et al., 2014; Husbands et al., 2007; Pereira et al., 2014). PthA4 directly induces the expression of *LOB1* by binding to the EBE region within its promoter, which designate it as a canker susceptibility (S) gene (Hu et al., 2014). An additional candidate, *CsSWEET1* is also regulated by PthA4 (Hu et al., 2014), but does not contribute to canker symptom development. The overexpression of *CsLOB1* was enough to induce canker-like pustules possibly by targeting a motif “GCGGCG” present in downstream genes (Duan et al., 2018; Zou et al., 2021). Knockout/Knockdown of *CsLOB1* by CRISPR and RNAi showed drastic reduction of citrus canker symptoms, demonstrating that this gene is essential to disease development (Huang et al., 2022; Jia et al., 2017; Jia et al., 2022a; Jia et al., 2022b; Jia and Wang, 2020; Zou et al., 2021). Although many

genes were upregulated by CsLOB1, none of the potential CsLOB1 downstream targets were subjected to further analysis (Duan et al., 2018; Zou et al., 2021).

Here, we characterize genes that are thought to act downstream of LOB1. We also demonstrate that a single *EXPANSIN* gene is a direct target of CsLOB1, but not PthA4. We have also identified the minimal promoter region required for its activation by CsLOB1 in *Citrus sinensis*. The *CsLIEXP1* can potentially be utilized as a target for genome editing to develop citrus canker resistant citrus varieties.

3.3. Results

In silico prediction of putative PthA4-regulated genes

Although *CsLOB1* is considered a master gene regulator induced by PthA4 during canker development (Duan et al., 2018; Zou et al., 2021), we hypothesized that PthA4 might directly activate the expression of other citrus genes involved in pustule formation. To test this hypothesis, transcriptomic data (Hu et al., 2014; Hu et al., 2016; Pereira et al., 2014) were used to identify genes that were highly up-regulated by PthA4 in addition to *CsLOB1* (Abe and Benedetti, 2016; Hu et al., 2014). In these transcriptomic analyses we selected genes which were highly upregulated. The promoter regions of the selected genes were aligned with the *CsLOB1*-EBE sequence (Hu et al., 2014) comparing primary and secondary nucleotides with high affinity to PthA4-RVD (Figure 1a) (Boch et al., 2009; Pereira et al., 2014). The EBE regions are usually close or overlapped with the TATA-box (Antony et al., 2010; Boch et al., 2009; Hu et al., 2014; Kay et al., 2007; Pereira et al., 2014; Römer et al., 2007). Consequently, 7 candidate genes were selected for further verification whether they are regulated by PthA4 (Figure 1, Table 1). These genes were classified into two biological processes: cell-wall remodeling and cell proliferation. In cell proliferation category, cyclin gene is associated with cell cycle (Cockcroft et al., 2000). Feruloyl-Coa-6 hydroxylase is a key enzyme to produce scopoletin/coumarin (Sun et al., 2015) and could be associated with cell proliferation (Abe and Benedetti, 2016). In cell-wall remodeling category, we found two pectate lyases and a polygalacturonase associated with pectin degradation of cell wall (Torki et al., 2000; Uluisik and Seymour, 2020). We also identified a lipid transfer protein, which mediates cell wall organization (Jacq et al., 2017), and an expansin coding gene, associated with cell-wall loosing (Cosgrove, 2000). All these genes contained a putative EBE sequence as the *CsLOB1*-EBE with high number of perfect matches and matches with second more frequent nucleotides (Figure 1b,c) (Abe and Benedetti, 2016). In addition, the predicted TATA-box sequences were overlapped with putative EBEs.

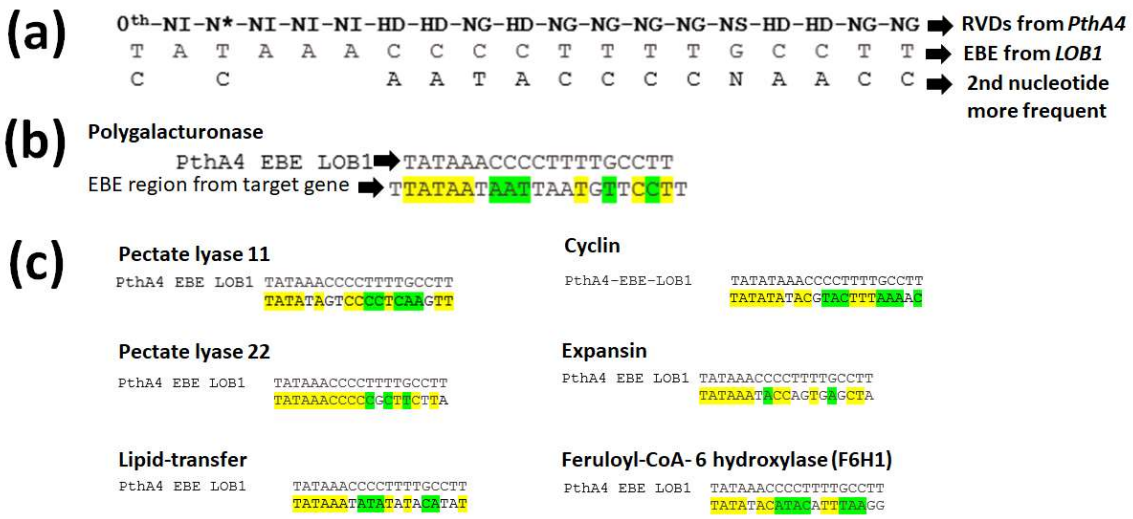


Figure 1. RVDs from PthA4 and putative EBEs from candidate susceptibility genes. (a) RVDs from PthA4, EBE from *CsLOB1*, and second nucleotide more frequent for RVD binding. (b) Example of alignment of *CsLOB1*-EBE with a putative EBE from a potential target gene. The sequences highlighted in yellow present a perfect match with *CsLOB1*-EBE. The sequences highlighted in green presented an alternative weaker match with the RVD of PthA4 at the same position. (c) Alignment of *CsLOB1*-EBE with other putative EBEs from candidate susceptibility genes.

Table 1. IDs and annotation of genes, and qPCR primers.

Phytozome	NCBI	HZAU	Annotation	Primer Forward	Primer Reverse
orange1.1g026556m	LOC102622391	Cs7g27640	LOB DOMAIN-CONTAINING PROTEIN 1-RELATED (LOB1)	TCGAGAAATGTGTTTTAGCTCCAT	AGACCATGCTGCTCACTGCAT
orange1.1g023962m	LOC102606716	orange1.1t00187	BETA EXPANSIN 6-RELATED	ATGCTCGGGAAATCCAGTGA	ACCCCAACGTTACGGAGTTG
orange1.1g027251m	LOC102618118	orange1.1t02532	POLYGALACTURONASE/PECTINASE	CCGGTGAGAGCCCAAACA	CAGGGCCACAAGCAACATTT
orange1.1g015623m	LOC102622327	Cs2g23970	PECTATE LYASE 11-RELATED	GGAGGGAAATCGCCAAAGAT	ATCACCGCATGCCTGAGAGT
orange1.1g023251m	LOC102612467	Cs5g06600	PECTATE LYASE 22-RELATED	TGGGTTCGGCAAGAATGC	TCACGAGCGAAAATGATCCA
orange1.1g027733m	LOC102629237	Cs9g13780	CYCLIN-U4-1	CATCATCTTCAGCTTGGAACCA	GATCTTTTGCGGGTGAAACC
orange1.1g034449m	LOC102623440	Cs5g07710	Probable lipid transfer (LTP_2)	CGTCACCATCGTGCTGTGA	CATTTGAAGTGACGAGGGACTCT
orange1.1g018077m	LOC102613413	Cs9g02930	feruloyl-CoA ortho-hydroxylase (F6H1)	GGATGATCCTGAAGTTGCAAAAT	TTCTTCTCCGCTGCTGACAA

Relative expression of candidate genes regulated by PthA4

Next, we tested if the candidate genes were indeed up-regulated by PthA4. Three different sweet orange varieties (Hamlin, Pineapple, and Valencia) were inoculated with wild type (*Xcc*-WT) and *pthA4* mutant *Xcc* (*Xcc*-Δ*pthA4*) with Phosphate-Buffered Saline (PBS) as control. The samples were collected 2 days after inoculation (DAI) and used for gene expression

analyses. As expected, *Xcc*-WT induced citrus canker symptoms and *Xcc*-Δ*pthA4* did not cause canker symptoms (Figure 2).

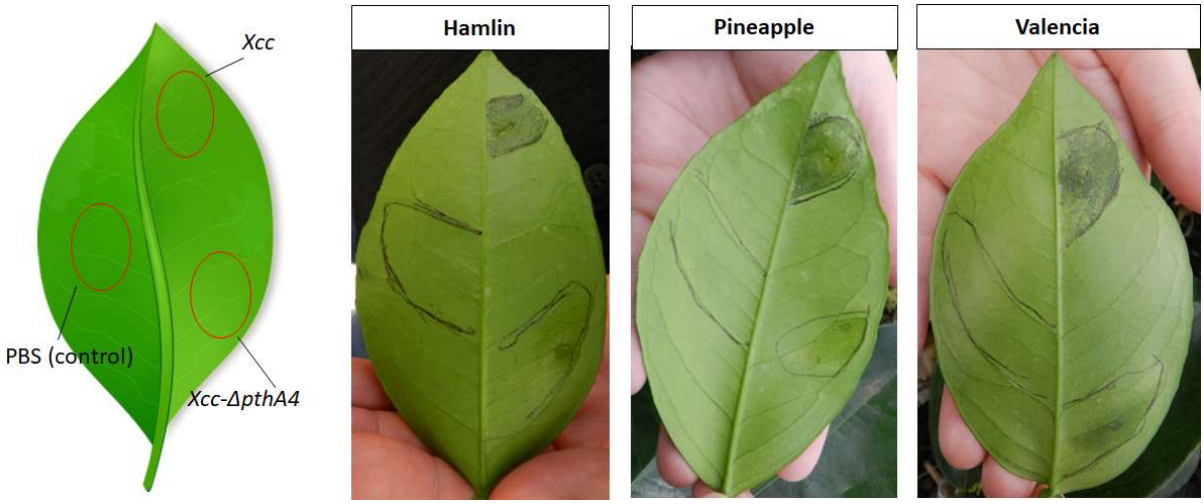
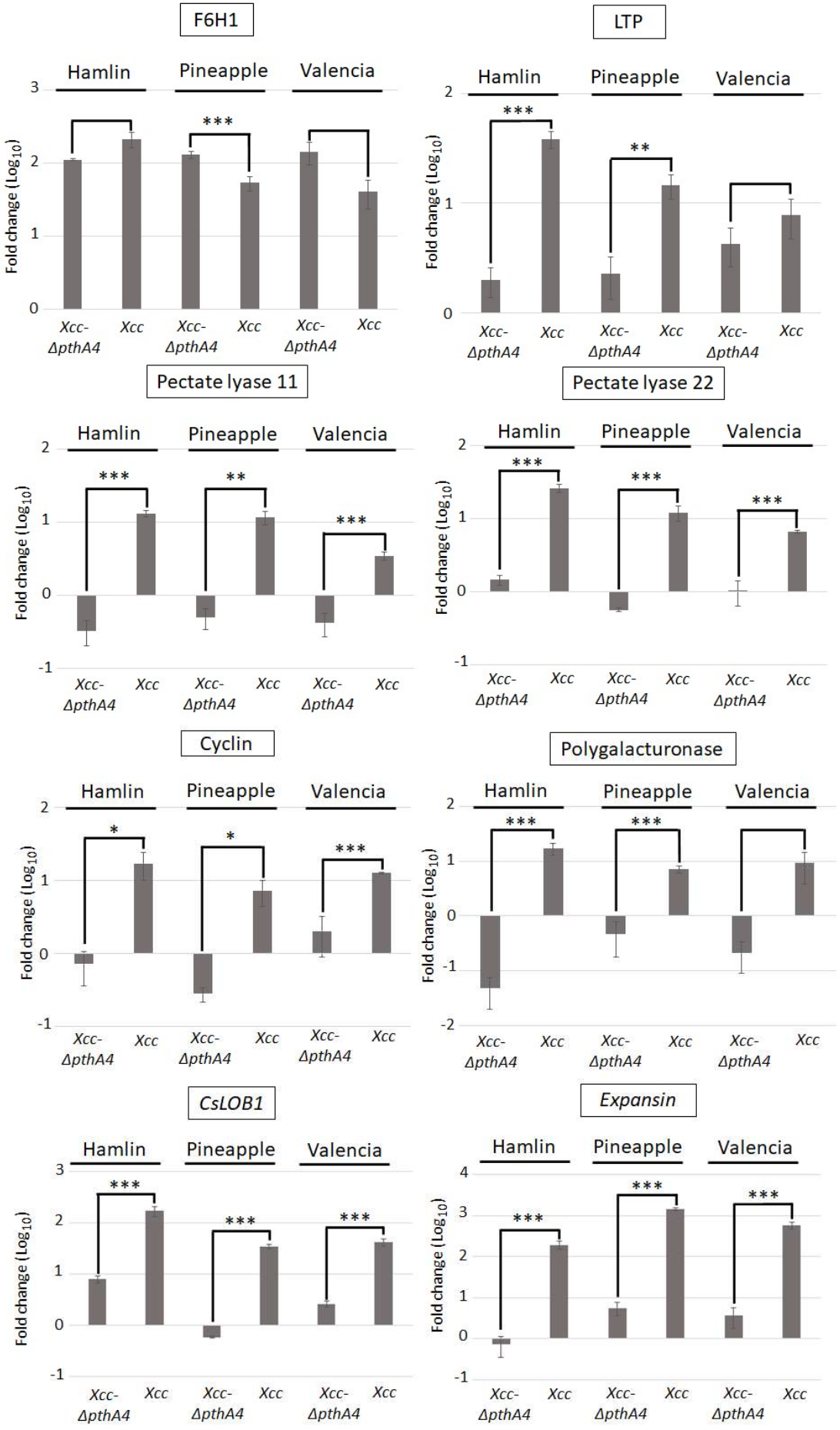


Figure 2. Citrus canker symptoms induced by wild type and *pthA4*-mutant of *Xcc*. The image to the left illustrates the inoculation areas in each leaf. The other images are citrus canker symptoms induced by *Xcc* and *Xcc*-Δ*pthA4* 14 DAI in different sweet orange genotypes (Hamlin, Pineapple, and Valencia).

RT-qPCR assays demonstrated that seven of the eight selected genes showed PthA4-dependent expression (Figure 3). *CsLOB1*, in particular, was induced by 50-100-fold (Figure 3). *Xcc* inoculation promoted the expression of *F6H1* (Figure 3). However, no difference was observed between *Xcc*-WT and *Xcc*-Δ*pthA4*, indicating that *F6H1* is not regulated by PthA4 (Figure 3). Curiously, cyclin, polygalacturonase and pectate lyase 11 coding genes were downregulated when infected with *Xcc*-Δ*pthA4* (Figure 3). The *EXPANSIN* gene was more induced by PthA4 than *CsLOB1*. The *EXPANSIN* gene induction varied from 300-1000-fold depending on the sweet orange genotypes (Figure 3).


F6H1
LTP
Pectate lyase 11
Pectate lyase 22
Cyclin
Polygalacturonase
CsLOB1
Expansin
Hamlin
Pineapple
Valencia
Fold change (Log10)
Xcc-ΔpthA4
Xcc
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**
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Figure 3. Relative expression of candidate susceptibility genes. Relative expression 48 hours post-inoculation with *Xcc* and *Xcc*-Δ*pthA4*. The treatments were compared to inoculation with phosphate-saline buffered (PBS). Statistical analyses were performed by Student's T-test. *: p <0.05; **: p <0.01; ***: p <0.001.

CsLOB1 induction by PthA3 through replacement of the EBE

Although *CsLOB1* was upregulated in all three sweet orange varieties evaluated, the relative expression level in Hamlin was higher than that in Valencia and Pineapple (Figure 3). Variations in the *LOB1* promoters were observed in different *Citrus* genotypes (Teper and Wang, 2021). The *CsLOB1* promoter region from those genotypes was further sequenced to identify allelic variations. We identified two alleles (Figure 4) which were previously described (Jia et al., 2017).

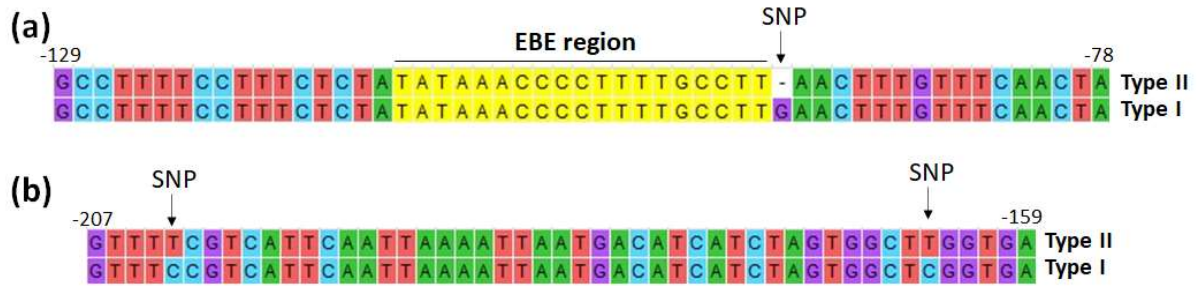

Figure 4. Genotyping of *CsLOB1* promoter. (a) Sequences of the EBE regions (highlighted in yellow) in the *CsLOB1* promoter. (b) Sequences of the *CsLOB1* promoter distant from EBE region. SNPs were identified in the promoter sequence. Genomic position referred to type I is demonstrated at the numbers on the top of beginning and end of sequences relative to the transcription start site.

Because induction of either *CsLOB1* allele is sufficient to induce canker symptoms (Jia et al., 2016), we used type II *CsLOB1* allele for further experiments. The *CsLOB1* native promotor was used to drive the GUS reporter (CsLOB1:GUS) (Figure 5a). *Agrobacterium tumefaciens* bacteria were transformed with binary constructs expressing *pthA4* or *pthA3* driven by the CaMV 35S promoter (35S:*pthA4* and 35S:*pthA3*). Using *A. tumefaciens* mediated transient expression in *Nicotiana benthamiana* leaves, we co-infiltrated CsLOB1:GUS with 35S:*pthA4* or 35S:*pthA3*. In each leaf, CsLOB1:GUS alone was infiltrated as a control of GUS basal expression (Figure 5b). High basal GUS activity was observed with CsLOB1:GUS alone (Figure 5c,d), probably owing to basal promoter activity. Higher GUS activity was observed when CsLOB1:GUS was co-introduced with 35S:*pthA4* (Figures 5c,d). No differences in GUS

activity were observed between co-infiltration of 35S:*pthA3* and CsLOB1:GUS compared to CsLOB1:GUS alone (Figures 5c,d). These results demonstrated that *CsLOB1* was directly induced by PthA4 as reported previously (Hu et al., 2014).

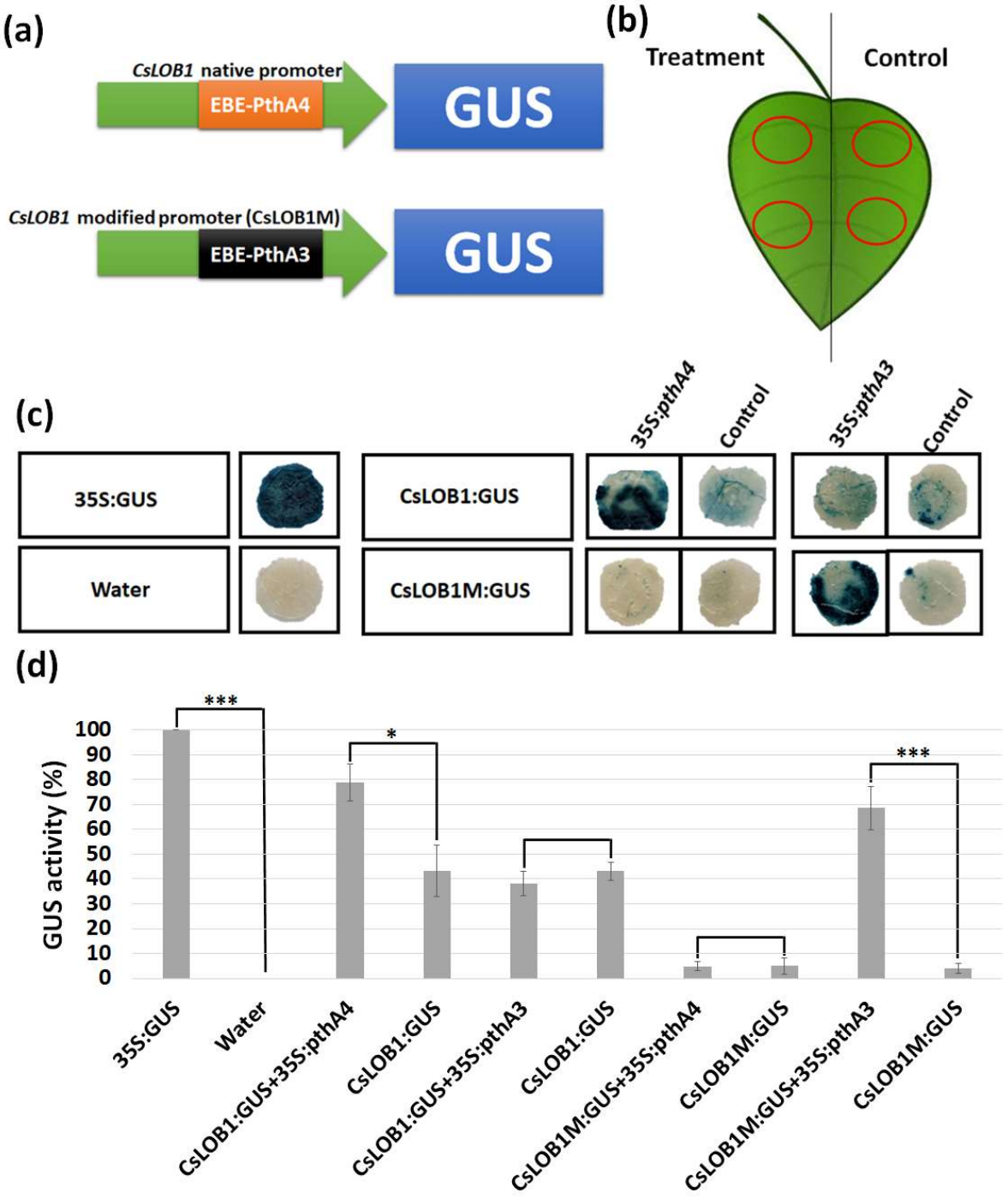

Figure 5. GUS assay to validate EBE replacement using *CsLOB1* promoter. (a) Schematic representation of the GUS promoter fusion constructs. *CsLOB1* native promoter fusion is represented as CsLOB1:GUS and modified *CsLOB1* promoter fusion is represented as CsLOB1M:GUS. CsLOB1M is the replacement of PthA4-EBE by PthA3-EBE. (b) Scheme of co-infiltration. The same leaf was infiltrated with the treatment (promoter:GUS and

35S:effector) and the respective control (promoter:GUS). (c) Histochemical GUS staining of co-infiltration treatments. 35S:GUS was used as a positive control of GUS activity. (d) Quantification of blue staining in leaf discs. Total areas of leaf discs were quantified using ImageJ software and compared with respective controls. The statistical analyses were performed by Student's T-test. *: $p < 0.05$; ***: $p < 0.001$.

The same approach described above was used to introduce a *CsLOB1* modified promoter to drive GUS reporter (CsLOB1M:GUS). EBE-PthA4 from this promoter was deleted and a predicted PthA3-EBE was introduced in the same position (Boch et al., 2009) (Figure 5a). Different from CsLOB1:GUS, the CsLOB1M:GUS control demonstrated lower basal levels of GUS activity (Figure 5c,d). This probably resulted from the removal of the TATA box overlapped with PthA4-EBE when EBE-PthA3 was cloned. Expectedly, GUS activity did not increase when CsLOB1M:GUS was co-infiltrated with 35S:*pthA4* (Figure 5c,d). Higher levels of GUS activity were detected for co-infiltration of 35S:*pthA3* and CsLOB1M:GUS (Figure 5c,d). These results indicated that PthA3-EBE can be introduced in promoter sequences to drive gene expression and be used as positive control in co-infiltration assays with 35S:PthA3.

An expansin gene is directly induced by CsLOB1 but not by PthA4

CsLOB1 is a transcription factor which was reported to induce an *EXPANSIN* gene expression (Duan et al., 2018; Zou et al., 2021). Because the differences in relative expression of the *EXPANSIN* gene among sweet orange genotypes (Figure 2) we sequenced its promoter regions (Figure 6). Curiously, the sequences were very conserved, and no differences were identified in the putative PthA4-EBE region and predicted *CsLOB1* binding site (Figure 6a,b). Thus, the differences in relative expression are owing to other factors. Two alleles were identified (type I and type II, Figure 6) based on two SNPs identified in the 600 bp upstream of the transcription start site (Figure 6c). Since the PthA4-EBE and predicted *CsLOB1* binding site sequences were conserved, we cloned the type I sequence to drive GUS expression in further experiments.

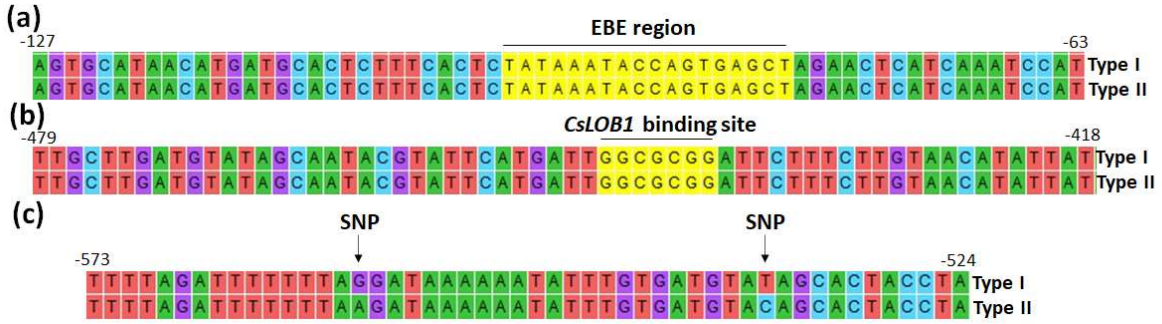

Figure 6. Genotyping of *EXPANSIN* promoter. (a) Sequences of *EXPANSIN* promoter surrounding the putative EBE region (highlighted in yellow). (b) Sequences of the *EXPANSIN* promoter surrounding the predicted *CsLOB1* binding site (highlighted in yellow). (c) Sequences of *CsLOB1* promoter distant from EBE region. Only these 2 SNPs were identified in the whole promoter region. Genomic position is demonstrated at the numbers on the top of beginning and end of sequences.

Although it was reported that CsLOB1 did not interact with the *EXPANSIN* gene promoter *in vitro* (Duan et al., 2018), no functional studies relating the expression of the *EXPANSIN* gene with *CsLOB1* and PthA4 have been published. Therefore, we used *A. tumefaciens* mediated transient expression in *N. benthamiana* leaves to evaluate these interactions. The *EXPANSIN* native promoter (EXP5) was used to drive GUS expression (Figure 7a). As controls, we produced two modified *EXPANSIN* promoters (Figure 7a). In the *EXPANSIN* modified promoter 1 (EXPM1), the PthA4-EBE was replaced by PthA3-EBE (Figure 7a). The *EXPANSIN* modified promoter 2 (EXPM2) contained a deletion of the predicted *CsLOB1* binding site (Figure 7a). All these constructs were co-infiltrated with 35S:*pthA4*, 35S:*pthA3* or 35S:*CsLOB1*. No GUS activity was detected in co-infiltration of EXP5:GUS and 35:*pthA4* or 35S:*pthA3* (Figure 7c,d). However, strong GUS activity was observed when EXP5:GUS was co-infiltrated with 35S:*CsLOB1* (Figure 7c,d), demonstrating that the *EXPANSIN* promoter is controlled by CsLOB1. These results indicated that *EXPANSIN* gene is not directly regulated by PthA4, but it is likely to be directly induced by CsLOB1. Therefore, we named the *EXPANSIN* gene as *CsLOB1-INDUCED EXPANSIN 1* (*CsLIEXP1*).

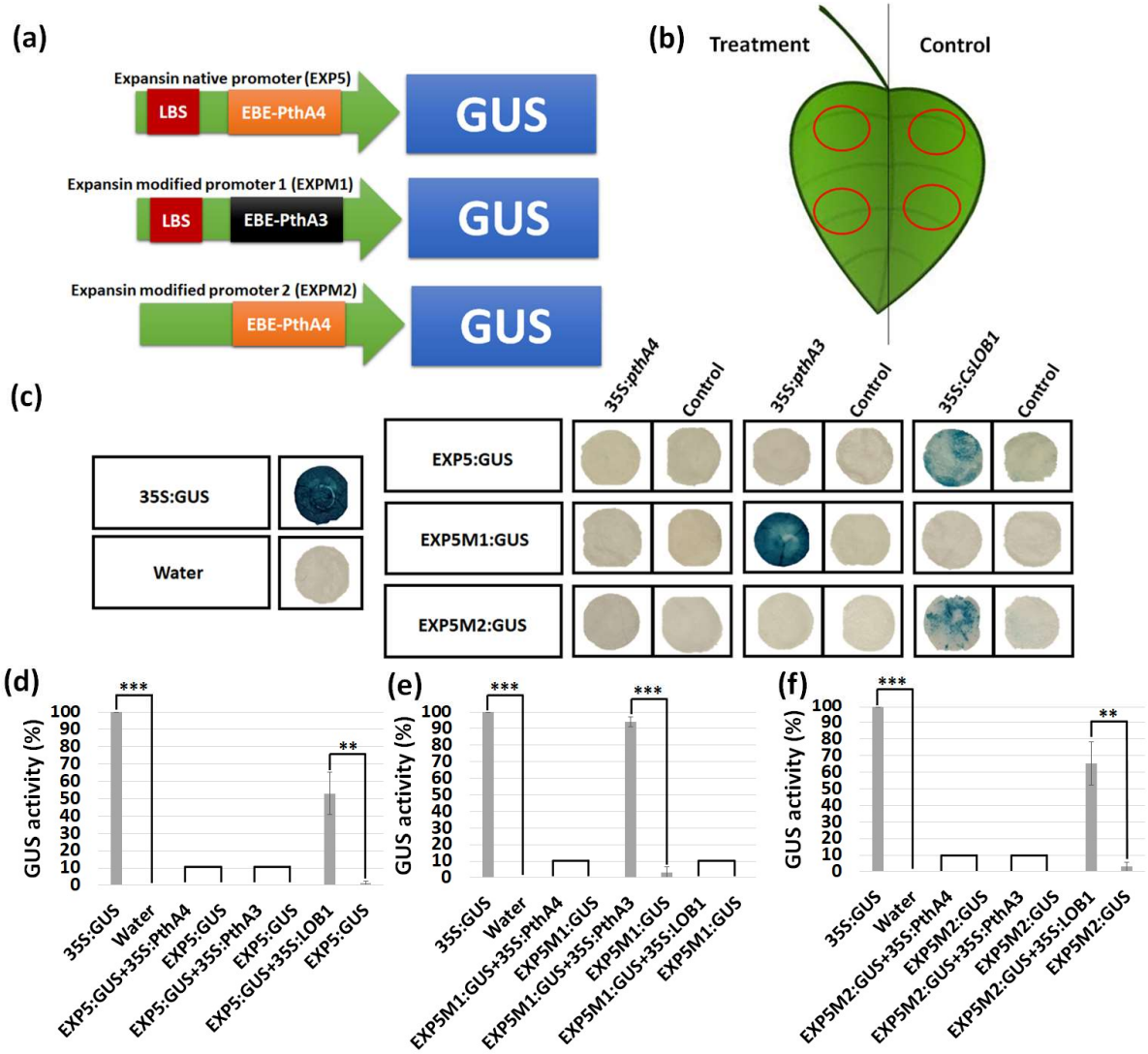

Figure 7. GUS assay of activation of *CsLIEXP1* promoters by PthA3, PthA4, and CsLOB1. (a) Schematic representation of the GUS promoter fusion constructs. EXP5 represents the native *CsLIEXP1* promoter. EXPM1 and EXPM2 represent modified *CsLIEXP1* promoters. LBS is a putative CsLOB1 binding site. EBE-PthA4 is a putative EBE for PthA4. EBE-PthA3 is a predicted EBE for PthA3 (Boch et al., 2009). EXPM1 is the modified promoter by replacement of PthA4-EBE with PthA3 EBE. EXPM2 is the modified promoter with LBS deleted. (b) Scheme of co-infiltration. The same leaf was infiltrated with the treatment (promoter:GUS and 35S:effector or 35S:*CsLOB1*) and the respective control (promoter:GUS). (c) Histochemical GUS staining of co-infiltration treatments. 35S:GUS was used as a positive control of GUS. (d), (e), and (f). Quantification of GUS activity in leaf discs in (c). Total areas of leaf discs were quantified using ImageJ software and compared with respective controls. The statistical analyses were performed by Student's T-test. **: $p < 0.01$; ***: $p < 0.001$.

EXPM1:GUS did not show GUS activity when co-infiltrated with 35S:*pthA4* (Figure 7c,e). High-level GUS activity was observed upon co-infiltration of 35:*pthA3* and EXPM1:GUS (Figure 7c,e), corroborating the result of the *CsLOB1* modified promoter (Figure 5c,d). Intriguingly, EXPM1:GUS co-infiltration with 35S:*CsLOB1* did not trigger GUS activity (Figure 7c,e), which probably resulted from the removal of the TATA-box. TATA-box is known to be critical for activation of gene expression (Huang et al., 2022; Kiran et al., 2006).

No GUS activity was detected when co-infiltrated EXPM2:GUS with 35S:*pthA4* and 35S:*pthA3* (Figure 7c,d). Surprisingly, strong GUS activity was detected when EXPM2:GUS was co-infiltrated with 35S:*CsLOB1* (Figure 7c,f). These data demonstrated that probably there are other unknown *CsLOB1* binding site in addition to the "GCGGCG" sequence reported previously (Duan et al., 2018; Zou et al., 2021).

Identification of *CsLIEXP1* minimum promoter regulated by CsLOB1

To establish the minimal promoter region required for *CsLIEXP1* induction by CsLOB1 and to narrow down regions that harbor potential CsLOB1 binding site, a series of truncated promoter regions of EXP5 (described in figure 8a) were cloned upstream of the GUS reporter. The promoters EXP1, EXP2, EXP3, EXP4 and EXP5 are, respectively, 130, 256, 392, 514, and 606 bp in length (Figure 8a). The constructs were co-infiltrated with 35S:*CsLOB1*. The expression of EXP1:GUS and EXP2:GUS was not induced by CsLOB1 (Figure 8b,c). Weak GUS activity was observed in co-infiltration of EXP3:GUS with 35S:CsLOB1 (Figure 8b,c). Thus, EXP3, which did not contain the predicated CsLOB1 binding site, was the *CsLIEXP1* minimum promoter regulated by CsLOB1, supporting the notion that there are other unknown CsLOB1 binding sites. Higher GUS activities were obtained in co-infiltration of EXP4:GUS or EXP5:GUS with 35S:CsLOB1 (Figure 8b,c), indicating the predicted CsLOB1 binding site is critical for activation of *CsLIEXP1*.

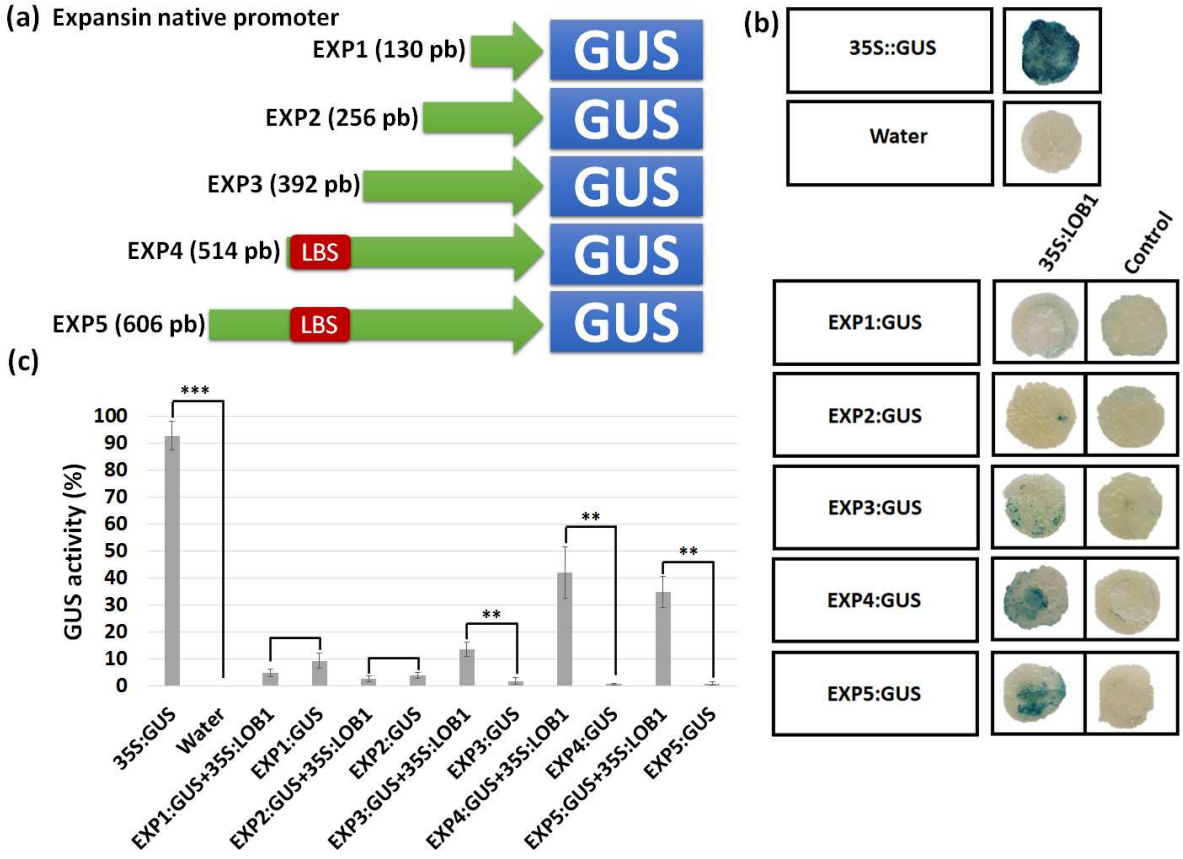

Figure 8. GUS assay to identify the minimum *CsLIEXP1* promoter induced by CsLOB1. (a) Schematic representation of the *CsLIEXP1* promoter truncations sizes (EXP1, EXP2, EXP3, EXP4, and EXP5) fused to a GUS reporter. LBS is a putative CsLOB1 binding site. (b) Histochemical GUS staining of co-infiltration treatments. 35S:GUS was used as a positive control of GUS. (c) Quantification of GUS activity in leaf discs. Total areas of leaf discs were quantified using ImageJ software and compared with respective controls. The statistical analyses were performed by Student's T-test. **: $p < 0.01$; ***: $p < 0.001$.

The *CsLIEXP1* gene is conserved in *Citrus sp*.

The amino acid sequence of CsLIEXP1 from *C. sinensis* was used to identify homologs in nine *Citrus* species and relatives. The CsLIEXP1 amino acid sequence was identical with that in *C. clementina*, and *C. ichangensis* (Figure 9). *C. medica* contained two identical copies of *CsLIEXP1* homolog with few differences from others (Figure 9). *C. maxima* had a deletion in the 5' CDS, which resulted in deletion of the first 36 amino acids at the N-terminus. However, the rest of sequence was identical to *C. clementina*, *C. sinensis* and *C. ichangensis*. Two different copies of *CsLIEXP1* homolog were identified in *Fortunella hindsii* genome, which were evolutionarily distant from *Citrus sp*., as well as sequences from *Poncirus*

trifoliata and *Atalantia buxifolia* (Figure 9). Although *A. buxifolia* and *P. trifoliata* sequences were more evolutionarily distant from *C. sinensis*, only few amino acids were different, but in general the sequences were very conserved, demonstrating that *LIEXP1* is conserved in *Citrus* and relatives.

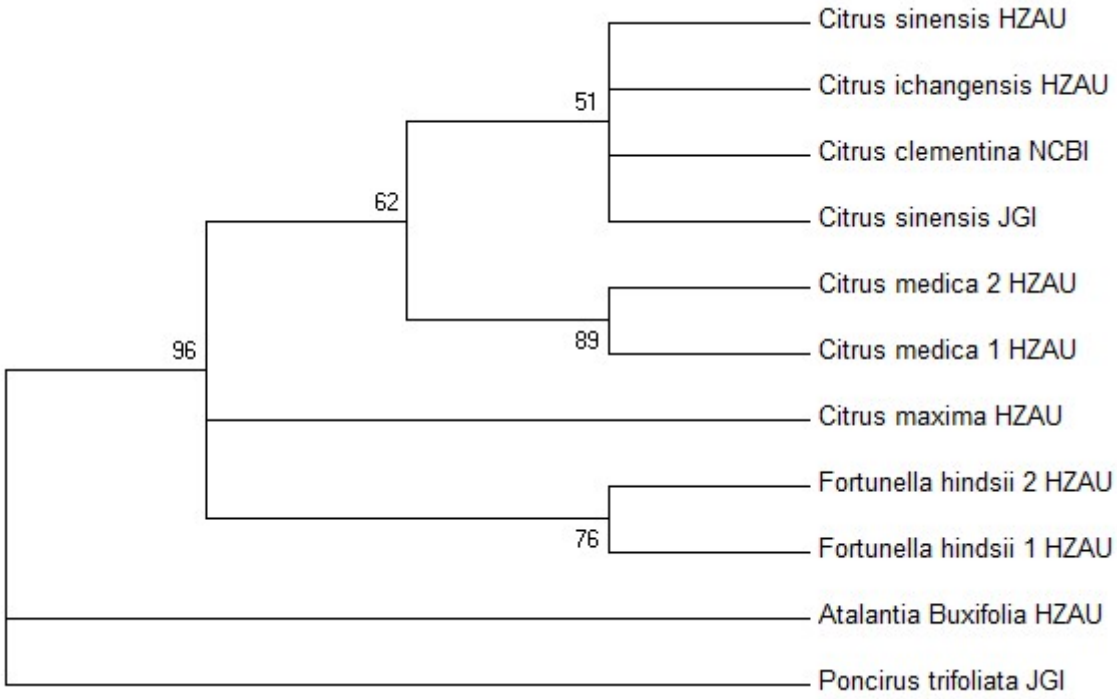

Figure 9. Phylogenetic analysis of *CsLIEXP1* in *Rutaceae*. Amino acid analysis was conducted using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The analysis involved 11 amino acid sequences. There was a total of 276 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 (Kumar et al., 2016).

The LOB domain and the *CsLIEXP1* promoter region are conserved in *Citrus sp*. and related genomes

The *LOB1* amino acid sequences (Figure 10) were aligned to identify the LOB domain. Using pFAM as reference, the LOB domain comprised 97 amino acids (Figure 10). The sequence begins with a proline at 80th position and ends with an alanine at 177th position in sweet orange (Figure 10). The LOB domain was completely conserved in the nine citrus genotypes analyzed (Figure 10). Two identical copies of *LOB1* were found in *C. medica* genome (Figure 10). Few variations were found in N- and C-terminus (Figure 10).

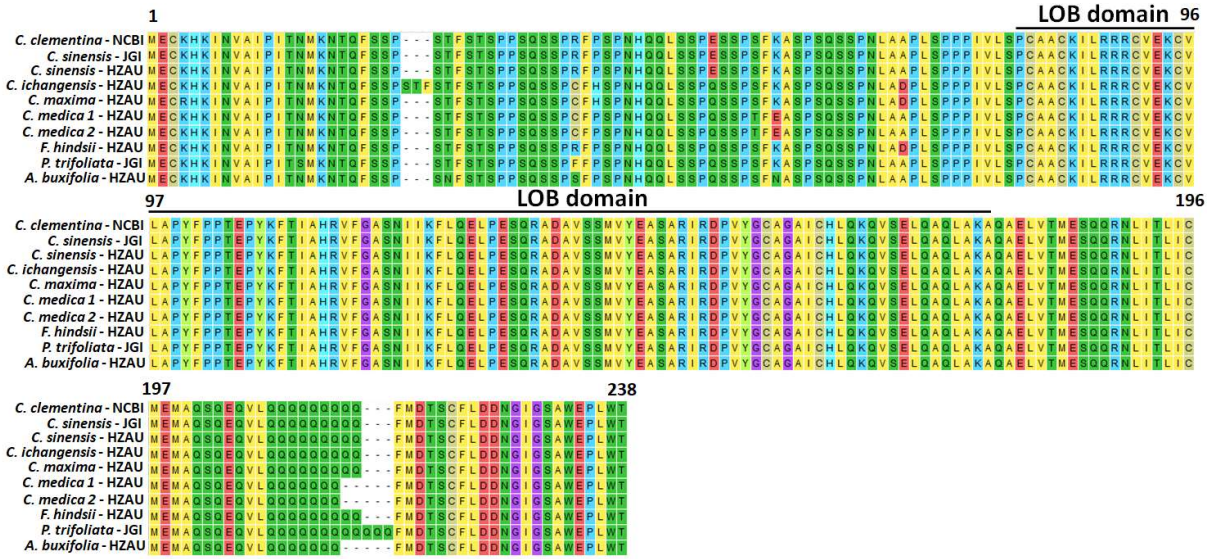

Figure 10. Multiple alignment of CsLOB1 amino acids in different citrus genotypes. In left side of sequences are the respective genotypes.

Since the LOB domain was conserved in different citrus genotypes, we examined variations in the promoter sequences of *LIEXP1*. The EXP1, EXP2 and EXP3 (Figure 8) sequences were conserved with few SNPs and indels (Figure 11). EXP3 was identified as the *CsLIEXP1* minimum promoter regulated by CsLOB1 (Figure 8). These results suggested that LOB1 could induce *CsLIEXP1* gene expression in other *Citrus* species and relatives. EXP4 and EXP5 sequences contained more variations compared to EXP1, EXP2 and EXP3 sequences, including the predicted *CsLOB1* binding site (Figure 11). Such variations in EXP4 and EXP5 could interfere with LOB1 binding and consequently affect *LIEXP1* gene expression in different *Citrus* genotypes.

Figure 11. Multiple alignment of the *CsLIEXP1* promoter in different citrus genotypes. The relationship between EXP1, 2, 3, 4, and 5 was demonstrated in Fig. 5A. The predicted CsLOB1 binding site is highlighted in yellow. In left side of sequences are the respective genotypes.

3.4. Discussion

CsLOB1 was previously described as the canker susceptibility gene regulated by PthA4 (Duan et al., 2018; Hu et al., 2014). TALEs containing two-to-nine mismatching-repeats to the EBE_{PthA4} were unable to induce CsLOB1 (Teper and Wang, 2021). In transcriptomic data, several sets of genes were upregulated by PthA4 besides *CsLOB1*. These include genes

associated to cell wall remodeling, and cell proliferation (Hu et al., 2014; Hu et al., 2016; Pereira et al., 2014). It remains to be determined whether any of the genes induced by PthA4 are direct targets of PthA4 in addition to *CsLOB1*. Based on transcriptomic data (Hu et al., 2014; Pereira et al., 2014) we identified seven upregulated genes associated with cell wall remodeling and cell proliferation with putative EBE similar to the EBE present in *CsLOB1* promoter. Interestingly, among the identified candidate genes, the levels of *CsLIEXP1* gene expression were even higher than that of *CsLOB1*, the known PthA4 target gene, in multiple citrus genotypes. The *CsLIEXP1* gene was the most induced gene among the evaluated ones in grapefruit overexpressing *CsLOB1* (Duan et al., 2018). In addition, *CsLIEXP1* was highly induced in sweet orange plants overexpressing *CsLOB1* (Zou et al., 2021). Although these studies suggested that *CsLIEXP1* gene is regulated by *CsLOB1*, no functional studies were performed to demonstrate this interaction. In this study, we evaluated the interaction of *CsLIEXP1* promoter with PthA4 and CsLOB1 by *A. tumefaciens* mediated transient expression in *N. benthamiana* leaves. Our analyses showed that the *CsLIEXP1* gene was only regulated by *CsLOB1* but not PthA4. In *Arabidopsis thaliana*, it was reported that LBD18 binds directly to *EXPANSIN 14* promoter, driving its expression (Kim and Lee, 2013), which supports our data. In tomato, two transcription factors were upregulated by the AvrBs3 TALE from *Xanthomonas gardneri,* which induces the expression of a pectate lyase gene and a pectinesterase gene that are directly associated with symptoms (Schwartz et al., 2017). Our results suggested that only CsLOB1 was direct target of PthA4, and other genes such as *CsLIEXP1*, upregulated in transcriptomes probably are likely downstream of CsLOB1 either directly or indirectly (Duan et al., 2018; Zou et al., 2021). In wheat, EBE-PthA4 replacement was used as positive control for GUS activation (Peng et al., 2019). Here, EBE-PthA3 was used as a positive control in GUS assay. It was the first time to functionally demonstrate the interaction of PthA3 with respective predicted EBE in an artificial system. These results lay the foundation to identify new genes regulated by PthA3 that can be associated with citrus canker disease, since PthA3 contributes to symptom development (Abe and Benedetti, 2016).

The *CsLOB1* binding motif "GCGGCG" described previously in *A. thaliana* and sweet orange (Duan et al., 2018; Husbands et al., 2007; Zou et al., 2021) was not found in the minimum *CsLIEXP1* promoter, suggesting other unknown motifs. ChIP-seq assays of *CsLOB1* binding motifs identified "GCGGCG" motif as a consensus sequence, however there were many different sequences binding with CsLOB1 in lower frequency (Zou et al., 2021). In *A. thaliana*, although "GCGGCG" motif was described as the LOB binding motif (Husbands et al., 2007), it was reported that the "GCGGCG" motif was not present in *EXPANSIN 14*

promoter, which was directly regulated by LBD18 (Kim and Lee, 2013). The large number of genes upregulated by PthA4 (Hu et al., 2014; Pereira et al., 2014), and the difference in expression levels could result from different interactions of CsLOB1 with these motifs and other transcription factors. Future studies are necessary to identify other unknown motifs. The *CsLIEXP1* promoter was very conserved until minimum promoter among different citrus species and relatives. The transient experiments in *N. benthamiana*, combined with the high PthA4-dependant expression of *CsLIEXP1* gene observed in three sweet orange varieties in this study demonstrated that this interaction was conserved. It is consistent with that overexpression of *CsLOB1* fused to glucocorticoid receptor (GR) in grapefruit (*C. paradise*) has shown the *LIEXP1* gene as the highest upregulated gene when treated with dexamethasone (DEX) (Duan et al., 2018). In Kumquat (*Fortunella hindsii*), the *LIEXP1* gene was induced in presence of PthA4, as well as LOB1, further supporting that the interaction is conserved (Teper et al., 2020).

Presumably, *CsLIEXP1* is involved in citrus canker pustule development by loosening the cell wall and promoting cell expansion (Cosgrove, 2015; McQueen-Mason and Cosgrove, 1995). Expansin proteins are translocated through the cell membrane and bind to the cell wall (Cosgrove, 2015; McQueen-Mason and Cosgrove, 1995). The plant cell wall is composed of layers of cellulose microfibrils directly interacting by noncovalent ligations with a matrix of pectins and xyloglucans (Cosgrove, 2015; McQueen-Mason and Cosgrove, 1995). It is possible that the direct binding of expansins to the cell wall breaks the noncovalent interactions without lytic activity, which results in loosening of the cell wall (Cosgrove, 2015; McQueen-Mason and Cosgrove, 1995). Our results suggest that the *CsLIEXP1* gene could be a putative susceptibility (S) gene indirectly induced by PthA4, which could be a target for genome editing. The editing of EBE and coding region of *CsLOB1* in sweet orange and grapefruit drastically reduced citrus canker symptoms (Huang et al., 2022; Jia et al., 2017; Peng et al., 2017). In other citrus species, Pummelo (*Citrus maxima*), 100% of editing was obtained for EBE region of *CsLOB1* resulting in abolishing of citrus canker symptoms (Jia and Wang, 2020). In rice, the editing of EBE and coding regions of S genes (*SWEET11*, *SWEET13*, *SWEET14*) promotes broad-spectrum resistance to *Xanthomonas oryzae* pv. *oryzae* (Oliva et al., 2019; Xu et al., 2019). These results demonstrated the efficiency of CRISPR/Cas9 technology to generate resistant crops based on genome editing of S genes modulated by TALEs. We envision that the genome editing of an indirect S gene by CRISPR/Cas9 technology could be a promising strategy to obtain resistant plants to *Xcc*. In sum, this is the first study that functionally demonstrated a gene directly induced by *CsLOB1*. This study suggests the

presence of novel CsLOB1 binding motifs and demonstrates *CsLIEXP1* as a potential target for gene editing to promote resistance to citrus canker.

3.5. Experimental procedures

In silico prediction of putative genes regulated by PthA4

Based on transcriptomic data (Hu et al., 2014; Pereira et al., 2014), we identified seventeen genes highly expressed and regulated by PthA4 (cut off ratio wild type/pthA4 mutant > 2). The sequences of 1500 bp nucleotides upstream of the transcription start sites of these genes were aligned with the *CsLOB1* EBE region using the software ClustalW (clustal.org/clustal2). PlantPan 2.0. software was used to predict putative TATA-box position. Genes that contained predicted EBE with high similarity to *CsLOB1* EBE, TATA-box overlapping, and sequences until 150 bp nucleotides downstream of transcription start site were selected as candidate genes for further investigation in this study.

Relative expression of putative genes regulated by PthA4 in different varieties of sweet orange

Five plants from each sweet orange variety (Pineapple, Hamlin and Valencia) were used to perform the experiments. Five leaves from each plant were inoculated with bacterial suspension of 10^5 cells/mL of *Xcc* wild type and *pthA4* deletion mutant (*Xcc*-Δ*pthA4*) (Abe and Benedetti, 2016). PBS buffer was used as negative control. Each leaf was inoculated with the three treatments. Three leaf discs were collected and pooled together from each plant for each treatment 48 hours after inoculation for assessing the relative gene expression. Two inoculated leaves from each plant were kept on the plant for 14 days to check symptoms. RNA was isolated from leaf discs using RNeasy Plant mini kit (Qiagen) following the manufacture's instructions. The samples were treated with DNase (Promega) and cDNA synthesis was performed using the GoScript™ Reverse Transcription System kit (Promega). The IDs of selected genes as well as sequences of primers for qPCR were described in Table 1. The qPCR primers were designed using Primer Express 2.0. software (Applied Biosystems). qPCR reactions were performed in duplicate using GoTaq® qPCR Master Mix (Promega) following the universal cycle protocol using ABI 7500 fast instrument (Applied Biosystems). *Citrus ACTIN 2* gene was used as the endogenous control for data normalization (Abe and Benedetti, 2016; Mafra et al., 2012). The results were calculated using ΔΔCT method (Livak and Schmittgen, 2001), comparing leaves inoculated with *Xcc* and *Xcc*-Δ*pthA4* to leaves inoculated with PBS buffer. Student's *t*-test was carried out to compare the differences in relative expression.

Genotyping of promoters of *CsLOB1* and *EXPANSIN* genes

Genomic DNA was isolated from the sweet orange varieties (Pineapple, Hamlin and Valencia) using the CTAB method. The promoter regions of *CsLOB1* and *EXPANSIN* genes were amplified from the three varieties using high fidelity enzyme and specific primers (Table 2). In both promoters we introduced a restriction site for *Hin*dIII in forward primer and *Xba*I for reverse primer (Table 2). The PCR products were applied to electrophoresis. The fragments were purified from gel using Wizard SV Gel and PCR Clean UP System kit (Promega). The 50 ng of purified fragments were cloned into pJET 1.2 vector using CloneJET PCR Cloning kit (Thermo Scientific). These constructs were transformed into *E. coli* DH10b strain by heat shock and cultivated for 90 minutes in SOC (2% tryptone (w/v), 0.5% yeast extract (w/v), 10 mM of NaCl, 2.5 mM of KCl, 10 mM of $MgCl_2$, 10 mM of $MgSO_4$, 20mM of Glucose) medium, then plated in LB medium supplemented with ampicillin (100 mg/L). The colonies were analyzed by PCR with primers provided by the CloneJET PCR Cloning kit (Thermo Scientific). Eight positive colonies for each promoter from each gene and for each variety were grown overnight and minipreps were carried out using QIAprep Spin Miniprep kit (Qiagen). The isolated plasmids were confirmed using Sanger sequencing and the sequences were aligned using MEGA7 software (Kumar et al., 2016).

Table 2. Primers for amplification of promoters.

Name primers	Primers Forward	Primer Reverse	Amplicon
EXP1	TATAAAGCTTGCACTACCTATTCATGATTGGC	TATAAAGCTTCTTTCGGTCGAAAAATGCATGC	130
EXP2	TATAAAGCTTCTCCTTGATATTTTTCCCCACC	TATAAAGCTTCTTTCGGTCGAAAAATGCATGC	256
EXP3	TATAAAGCTTTGTAAAGGAAATCTATGTAAGA	TATAAAGCTTCTTTCGGTCGAAAAATGCATGC	392
EXP4	TATAAAGCTTCTTTCGGTCGAAAAATGCATGC	TATAAAGCTTCTTTCGGTCGAAAAATGCATGC	514
EXP5	AAGCTTCTAATTAAACTAAATGTATT	TATAAAGCTTCTTTCGGTCGAAAAATGCATGC	606
CsLOB1 (promoter)	AAGCTTGAAAGTACATCCATAACC	TCTAGAGGTTGTAGTAGTTAGTGAGA	575
CsLOB1 (CDS)	TATATCTAGAATGGAATGCAAACACAAAATT	TATAGAGCTCTCATGTCCACAGAGGCTCCCAA	734

Cloning of promoters into binary vector to drive GUS expression

The pBI121 vector contains a CaMV (Cauliflower Mosaic Virus) 35S promoter driving the *uidA* gene that encodes a β-glucuronidase (GUS). This plasmid was used as backbone to introduce the *EXPANSIN* (different sizes) and *CsLOB1* promoters (Table 2). The promoter fragments were amplified using high fidelity DNA polymerase and specific primers (Table 2). PCR products were applied to electrophoresis. The sequences were purified from gel using Wizard SV Gel and PCR Clean UP System kit (Promega). The pBI121 vector was digested with *Hin*dIII and *Xba*I restriction enzymes to remove the 35S promoter from the plasmid and purified as described above. The purified PCR products were digested with the same enzymes, purified as described above, and ligated into the pBI121 vector. Each promoter was individually introduced into pBI121 to drive GUS expression. The ligation product was transformed into *E. coli* Dh5α strain. Positive colonies were cultivated overnight in LB medium supplemented with kanamycin (100 mg/L) and minipreps were performed using a QIAprep Spin Miniprep kit (Qiagen). The plasmids were confirmed using Sanger sequencing. The whole process of cloning described here was also applied to synthesized fragments by GenScript. These synthetic sequences were based on *CsLOB1* and *EXPANSIN* native promoters but with some modifications. The first one is the PthA4-EBE for *CsLOB1* which was replaced by the predicted PthA3-EBE, designated as *CsLOB1M*. The predicted PthA4-EBE for *EXPANSIN* was replaced by the predicted PthA3-EBE, named as EXPM1. Another synthetic sequence for *EXPANSIN* promoter was based on the native promoter with a deletion for the predicted *CsLOB1* binding site, named as EXPM2. The CsLOB1 binding site "GGCGCGG" was predicted as described previously (Zou et al., 2021). All the synthetic sequences were introduced into pBI121 to drive GUS expression as aforementioned and confirmed using Sanger sequencing. We also cloned the 35S promoter to drive *CsLOB1* gene expression. Specific primers (Table 2) were designed to amplify the *CsLOB1* gene, with restriction site for *Xba*I in forward primer and *Sac*I in reverse primer. *CsLOB1* was amplified using a high-fidelity enzyme from cDNA of Valencia sweet orange 48 hours after infection with *Xcc*.

Transient expression in *Nicotiana benthamiana*

All vectors, including 35S:PthA3 and 35S:PthA4 (Domingues et al., 2010), were transformed into *Agrobacterium tumefaciens* GV2260 strain by electroporation. One positive PCR colony of each construct was cultivated overnight in YEP medium (yeast extract 1% (w/v), NaCl 0.5 % (w/v), peptone 0.5% (w/v), agar 0.7% (w/m), pH 7.0) with rifampicin (50 mg/L) and kanamycin (100 mg/L) at 28 °C and 180 rpm. The bacteria were centrifuged at 5000 rpm

for 10 minutes and suspended in 2 mL of agroinfiltration buffer (10 mM $MgCl_2$, 10 mM MES, 0.2% sucrose (w/v), 0.2 mM of acetoseringone, pH 5.6) and incubated for 4 hours at 28 °C and 180 rpm. The ODs were adjusted to 0.2 (1:1) in the combinations described in Table 3 and they were infiltrated into leaves of 6-week-old *Nicotiana benthamiana* plants using a needless syringe. *N. benthamiana* cultivated in 22 °C, with 16 hours of light and 50% humidity. As negative control, the *Agrobacterium* carrying only the promoter:GUS constructs were also infiltrated in the same leaves of the treatments. Leaf discs with 1 cm of size were collected three days after inoculation for GUS histochemical assay. In GUS histochemical assay the samples were incubated with X-Gluc solution overnight 37 °C. The leaf discs were distained with ethanol for pictures. Four replicates were used for each treatment. The blue area of each leaf disc was individually and manually calculated using the software ImageJ and used to calculate the percentage from leaf disc total area. The statistical analyses were performed by Student's T-test.

Table 3. Combinations of *A. tumefaciens* transformed with pBI121 for co-infiltration experiments.

Experiment #1		Experiment #2		Experiment #3	
Inoculum 1	**Inoculum 2**	**Inoculum 1**	**Inoculum 2**	**Inoculum 1**	**Inoculum 2**
CsLOB1::GUS	**35S::PthA4**	**EXP-5::GUS**	**35S::PthA4**	**EXP-1::GUS**	**35S::CsLOB1**
CsLOB1::GUS	**35S::PthA3**	**EXP-5::GUS**	**35S::PthA3**	**EXP-2::GUS**	**35S::CsLOB1**
CsLOB1M:: GUS	**35S::PthA4**	**EXP-5::GUS**	**35S::CsLOB1**	**EXP-3::GUS**	**35S::CsLOB1**
CsLOB1M:: GUS	**35S::PthA3**	**EXPM1::GUS**	**35S::PthA4**	**EXP-4::GUS**	**35S::CsLOB1**
		EXPM1::GUS	**35S::PthA3**	**EXP-5::GUS**	**35S::CsLOB1**
		EXPM1::GUS	**35S::CsLOB1**	**EXPM1::GUS**	**35S::CsLOB1**
		EXPM2::GUS	**35S::PthA4**		
		EXPM2::GUS	**35S::PthA3**		
		EXPM2::GUS	**35S::CsLOB1**		

Phylogenetic tree of the *CsLIEXP1* gene in different citrus genotypes

The amino acid sequence of the *CsLIEXP1* gene from *C. sinensis* was used to BLAST against 8 Citrus genotypes including *Citrus sinensis*, *Citrus clementina*, *Citrus*

ichangensis, *Citrus medica*, *Citrus maxima*, *Fortunella hindsii*, *Atalantia buxifolia*, and *Poncirus trifoliata*. The sequences were aligned using ClustalW algorithm by MEGA7 software (Kumar et al., 2016). This software was also used to create a phylogenetic tree using maximum likelihood method with a bootstrapping procedure of 1000 repetitions.

Alignment of *CsLIEXP1* promoter regions

The eight genotypes for phylogenetic tree analysis were used for characterization of the *EXPANSIN* promoter. The alignment was performed with 11 sequences identified using 1500 nucleotides upstream of the *CsLIEXP1* gene transcription start site. The software MEGA7 (Kumar et al., 2016) was used to align the sequences by ClustalW algorithm.

Alignment of CsLOB1 amino acids and domain identification in different citrus genotypes

The *CsLOB1* amino acid sequence from *C. sinensis* was applied in InterProScan tool (Quevillon et al., 2005) to identify LOB domain position. The *CsLOB1* sequences from the eight genotypes described above were aligned with the *C. sinensis* var. Pineapple sequence using ClustalW algorithm from MEGA7 software (Kumar et al., 2016). The LOB domain was compared among the sequences.

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4. Chapter 2: Application of CRISPR/Cas9 technology for gene editing of CsLIEXP1 and its role in citrus canker development

4.1. Abstract

CRISPR/Cas has been used as a tool for gene editing of many plants. However, the screening of edited plants sometimes could be a challenge depending on genomic sequence. High Resolution Melting Analysis (HRMA) is a very accurate technique that could identify even one different nucleotide between sequences and accelerate screening of edited plants. In citrus, it was reported that *CsLOB1* is a transcription factor and a susceptibility (S) gene modulated by PthA4 effector from *Xanthomonas citri* subsp. *citri* (*Xcc*). The genome editing of *CsLOB1* promoted citrus canker resistance. The *CsLIEXP1* is directly regulated by CsLOB1 and a susceptibility gene regulated by PthA4. Here, sweet orange transgenic plants expressing Cas9 and CsLIEXP1_sgRNAs were obtained. These plants were screened by HRMA, which is a powerful technique that identified in our study, even 10% of edited sequences. One transformed plant was selected, and the DNA edited was confirmed by Sanger sequencing. This plant presented 43.75% of *CsLIEXP1* edited sequences with two types of modification, a deletion of a thymine and a deletion of five nucleotides. This edited plant was resistant to *Xcc* even with 43.75% of edited sequences, which demonstrated that *CsLIEXP1* contributes for

citrus canker symptoms development. This was the first study to show an expansin edited plant, which was more resistant to citrus canker disease.

4.2. Introduction

CRISPR/Cas is a powerful technology for genome editing (Jinek et al., 2012). This technology has been widely used for gene editing of many plants, such as *Arabidopsis thaliana*, tobacco, rice, sorghum, watermelon, banana, potato, soybean, maize, apple, citrus and other (Jia and Nian, 2014; Jiang et al., 2013; Kaur et al., 2018; Li et al., 2015; Liang et al., 2014; Nerkar et al., 2022; Nishitani et al., 2016; Wang et al., 2015; Wang et al., 2021).

Identification of edited plants could be difficult because sequencing is necessary to validate the gene editing, which could spend too much time and money depending on the number of samples. Thus, methods for screening of edited plants could be an alternative for fast selection of edited-candidate samples. A common method that has been widely used for screening of edited plants is based on restrict enzyme assay (Nadakuduti et al., 2019; Zhang et al., 2017). In this method, the sgRNA is designed overlapped to a restriction enzyme site closed to PAM sequencing, once Cas9-induced mutations normally are 2-3 nucleotides from PAM sequence (Zhang et al., 2017). Thus, when Cas9 induce mutations in this region, a frameshift is generated and restriction enzyme cannot bind and cleave the sequence anymore, and the edited-plants can be selected by band size difference in electrophoresis (Zhang et al., 2017). However, some genomic regions did not have PAM sequence overlapped with restriction enzymes, and the High Resolution Melting Analysis (HRMA) could be an alternative method for screening of edited plants (Chen et al., 2018; Denbow et al., 2017). This is a qPCR technique initially used for genotyping and recently employed to gene editing screening in many organisms including plants (Carrington et al., 2022; Housden and Perrimon, 2016; Kojin et al., 2021; Pham et al., 2020; Ren et al., 2013). It is a very sensitive technique based on melting curve able to sense even one nucleotide of difference between sequences and to be performed in any genomic region (Chen et al., 2018; Denbow et al., 2017). In citrus, this technique was not tested yet, but could accelerate the screening of multiple sgRNA.

In citrus, it has been reported the editing of *CsLOB1*, a susceptibility (S) gene and direct target for PthA4 effector from *Xanthomonas citri* subsp. *citri* (*Xcc*) (Duan et al., 2018; Hu et al., 2014; Zou et al., 2021). The *pthA4* gene belongs a Transcription Activator-Like Effector (TALE) class (Boch et al., 2009). Thus, PthA4 induces the *CsLOB1* expression, which one is a transcription factor (Abe and Benedetti, 2016; Duan et al., 2018; Hu et al., 2014). CsLOB1 induces expression of genes associated with cell proliferation and cell wall

remodeling, which contribute to citrus canker symptoms (Duan et al., 2018; Zou et al., 2021). The *CsLOB1*-edited plants were resistant to citrus canker demonstrating that editing of S genes is a promise strategy to obtain disease-resistant plants (Jia et al., 2017; Jia et al., 2022b; Jia et al., 2022a; Jia and Wang, 2020; Peng et al., 2017). In rice, this approach has been successfully used for editing of three S genes associated to sugar transport, *SWEET11*, *SWEET13* and *SWEET14*, which presented resistance to *Xanthomonas oryzae* (Oliva et al., 2019; Xu et al., 2019). In citrus, we showed that an expansin gene is directed regulated by CsLOB1, therefore indirectly induced by PthA4 (de Souza-Neto, data not published yet). This gene was named *CsLOB1-INDUCED EXPANSIN 1* (*CsLIEXP1*) and considered a putative S gene indirectly induced by PthA4 (de Souza-Neto, data not published yet). The expansin genes are associated to no-lytic cell wall loosening, which promotes cell expansion (Cosgrove, 2000; Cosgrove, 2015; McQueen-Mason and Cosgrove, 1995). Cell expansion and proliferation are the key phenotypes associated to citrus canker disease (Duan et al., 2018; Zou et al., 2021), which suggests that *CsLIEXP1* gene could contributes to disease development. Here, we showed that HRMA can be a powerful tool for screening of edited sweet orange plants. In addition, we proved that expansin is a S gene important for citrus canker development, since expansin-edited plant by CRISPR/Cas9 showed less symptoms of citrus canker disease.

4.3. Results

Design of sgRNAs and genetic transformation of sweet orange with pDIRECT_CsLIEXP1 vector

The *CsLIEXP1* first exon from Hamlin variety of sweet orange was amplified by PCR and cloned in pJET1.2. (Thermo Scientific). Thirty colonies were sequenced and only one allele was identified in the *CsLIEXP1* first exon (Figure 1).

Figure 1. Genotyping of *CsLIEXP1* first exon. Thirty sequences of *CsLIEXP1* first exon were sequenced and only one allele was obtained. Numbers on top of sequences represented the genomic position compared to *CsLIEXP1* transcription start site.

Based on these results we designed 3 sgRNAs (Figure 2a). The CsLIEXP1_gRNA1 was designed in complementary sequence differently from the CsLIEXP1_gRNA2 and CsLIEXP1_gRNA3 which were designed in forward sequence (Figure 2a). The total distance

between the most distant sequences were 78 nucleotides (Figure 2a). These sgRNAs were introduced in pDIRECT22C following specific protocol (Čermák et al., 2017). The final construction is demonstrated in figure 2b.

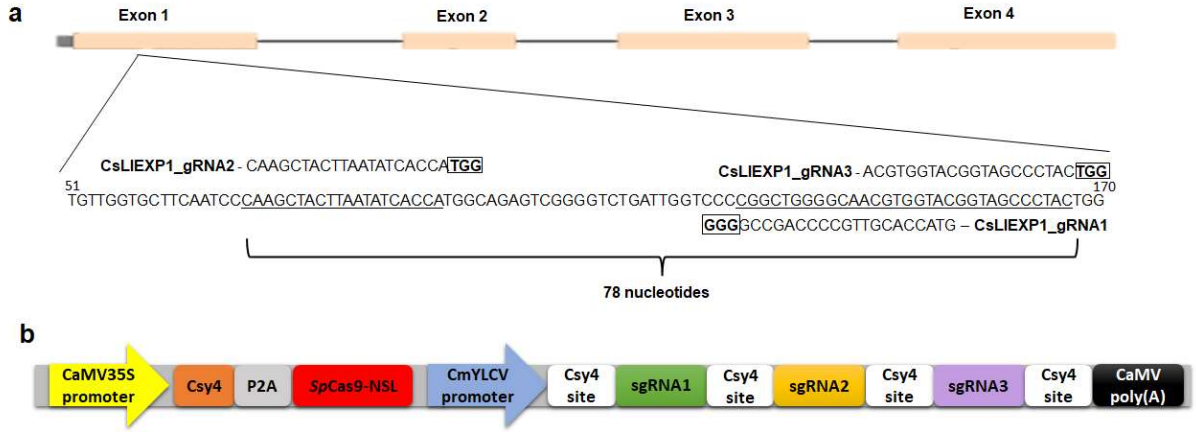

Figure 2. Design and construction of pDIRECT_CsLIEXP1. (a) Design of sgRNAs in *CsLIEXP1* first exon. Each sgRNA is individually demonstrated at the figure. Gene structure was adapted from <https://phytozome-next.jgi.doe.gov>. The PAM sequences were highlighted in upstream sequence. The total distance of beginning and end of sgRNAs is 78 nucleotides. (b) The final construction of pDIRECT_CsLIEXP1.

Four genetic transformation experiments were performed (Table 1). We infected 3033 explants with *Agrobacterium tumefaciens* transformed with pDIRECT_CsLIEXP1 and obtained 342 shoots (Table 1). DNA was extracted from these shoots and used to perform PCR with specific primers (Čermák et al., 2017). A total of 22 samples were positive PCR for genetic transformation (Table 1).

Table 1. Organization of genetic transformation experiments.

Experiment	Explants	Shoots	Positive PCR	Efficiency
#1	635	64	8	12.5%
#2	774	80	8	10%
#3	984	96	4	4.2%
#4	644	84	2	2.4%
Total	3033	342	22	6.5%

High Resolution Melting Analysis for screening of edited plants

High Resolution Melting Analysis (HRMA) was reported as a method for screening of edited plants by CRISPR/Cas9 (Chen et al., 2018; Denbow et al., 2017). In the most cases of citrus genome editing, the plants were not completely edited, which became a chimera with

wild type and edited cells (Jia et al., 2016; Jia et al., 2017; Peng et al., 2017). The first exon sequence of *CsLIEXP1* was used to check whether HRMA could identify plants with different proportions of edited sequences. Wild type and thymine mutant (previously obtained) *CsLIEXP1* sequences were mixed in different ratio and applied to HRMA. Although the software identified the 10% of edited sequences as the same variant with wild type (0% of edited sequences), a small deformation of melting curve is observed (Figure 3). This shift in melting curve increased when the percent of edited sequences was increased as well (Figure 3). Even with 40% percent of edited sequences the software recognized this sample as the same variant of wild type (Figure 3). However, the sample with 50% of edited sequences was identified as a new variant by the software (Figure 3). These results possibly can be explained because this software was initially developed for genotyping, which normally is 100% or 50% of identical sequences. The melting curve shape changes were more evident in samples from 50% until 80% of edited sequences, but the software still identified as the same variant (Figure 3). Samples with 90% and 100% of edited sequences were identified as two new variants by the software (Figure 3). These results demonstrated that any deformation in melting curve identified by HRMA could indicate an edited plant. The results also demonstrated that samples identified as different variant from wild type could indicate 50% or more edited sequences.

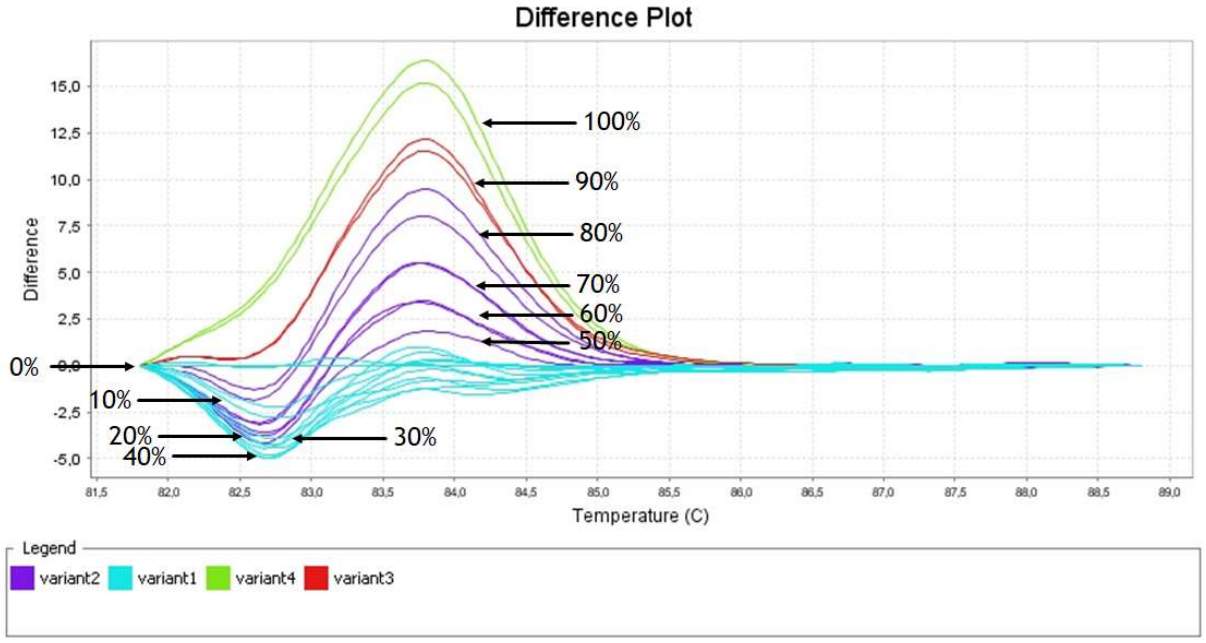

Figure 3. Test for HRMA accuracy. 0 to 100% is a range of mixed wild type and edited sequences, which the percentage represented the ratio of edited sequences.

The validation of HRMA using samples with specific concentration of mixed sequences allowed us to apply this technique for transgenic plants previously obtained. DNA

from transformed plants with pDIRECT_CsLIEXP1 were used to perform HRMA. One transgenic plant was selected as candidate edited plant based on small deformation identified in melting curve by HRMA (Figure 4). The melting curve of sample and wild type were very similar and included as the same variant by the software (Figure 4).

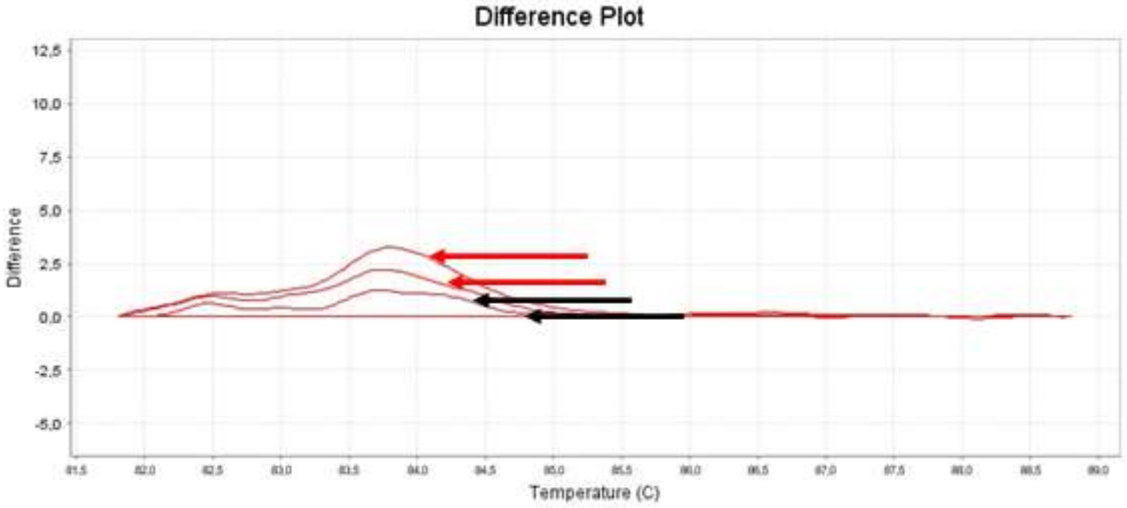

Figure 4. HRMA for H_CsLIEXP1_1. Black arrows indicated the replicates of wild type sample. The red arrows indicated replicates of the H_CsLIEXP1_1.

Identification of edited *CsLIEXP1* sequences in Cas9-expressing transgenic plants

The *CsLIEXP1* target sequence from selected plant by HRMA were amplified by PCR with high fidelity DNA polymerase using the HRMA primers. The PCR products were applied to electrophoresis, and the amplicons were purified from gel. These sequences were cloned in pJET1.2 (Thermo Scientific) and transformed in *E. coli*. The plasmids were confirmed by Sanger sequencing. We identified only two types of gene editing, the first one was a deletion of five nucleotides guided by CsLIEXP1-gRNA1 (Figure 5a). The other type of gene editing was a deletion of a thymine guided by CsLIEXP1-gRNA3 (Figure 5b). No deletions from one sgRNA to another were obtained and no gene editing were guided for CsLIEXP1-gRNA2. Deletion of thymine was the most frequent observed in the sample than deletion of five nucleotides (Figure 5). The editing frequency observed in H_CsLIEXP1_1 was 43.75% of gene editing, which corroborated melting curve in HRMA. These data validated HRMA as a powerful technique for screening of edited plants.

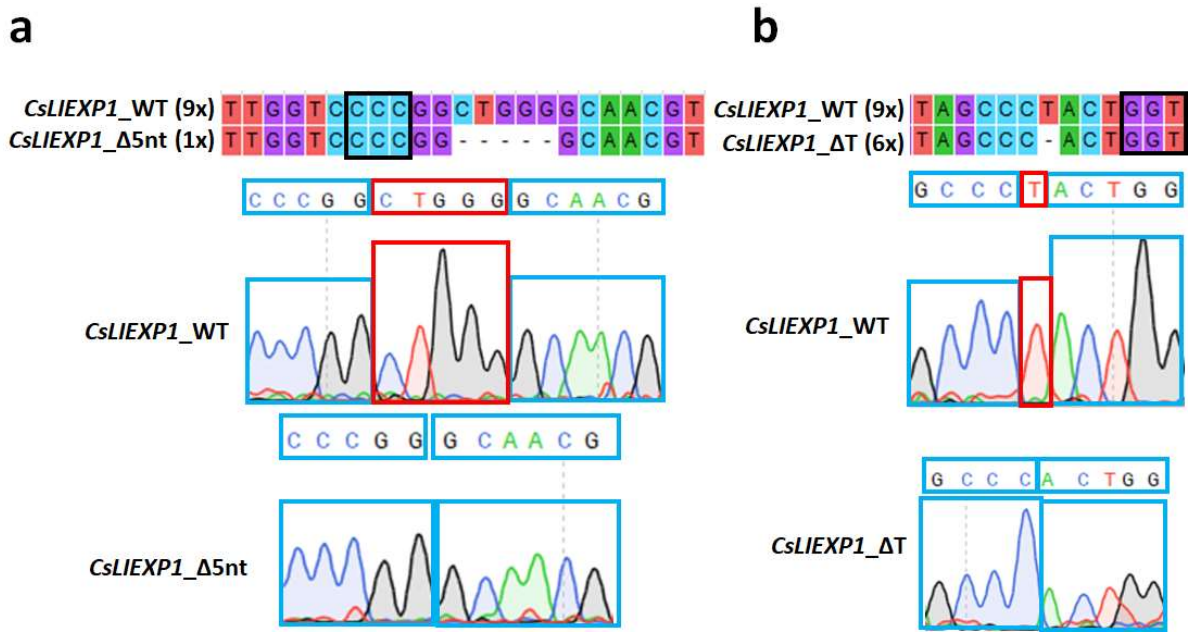

Figure 5. Sequencing of H_CsLIEXP1_1 edited plant. (a) Sequencing of *CsLIEXP1* target sequencing for CsLIEXP1-gRNA1. (b) Sequencing of *CsLIEXP1* target sequencing for CsLIEXP1-gRNA3. On the top it is demonstrated sequence alignment. On the bottom, representative electropherograms for each sample. In electropherograms, the up and downstream sequences of edited regions are represented in blue, and the deleted sequences were represented in red.

Other transgenic line was selected by HRMA as different variant from wild type and confirmed by Sanger sequencing (Figure 6). This sample presented 93.75% of edited sequences with the same types of modification identified in figure 5. However, this plant did not survive the grafting, and because that the experiments were conducted only with H_CsLIEXP1_1.

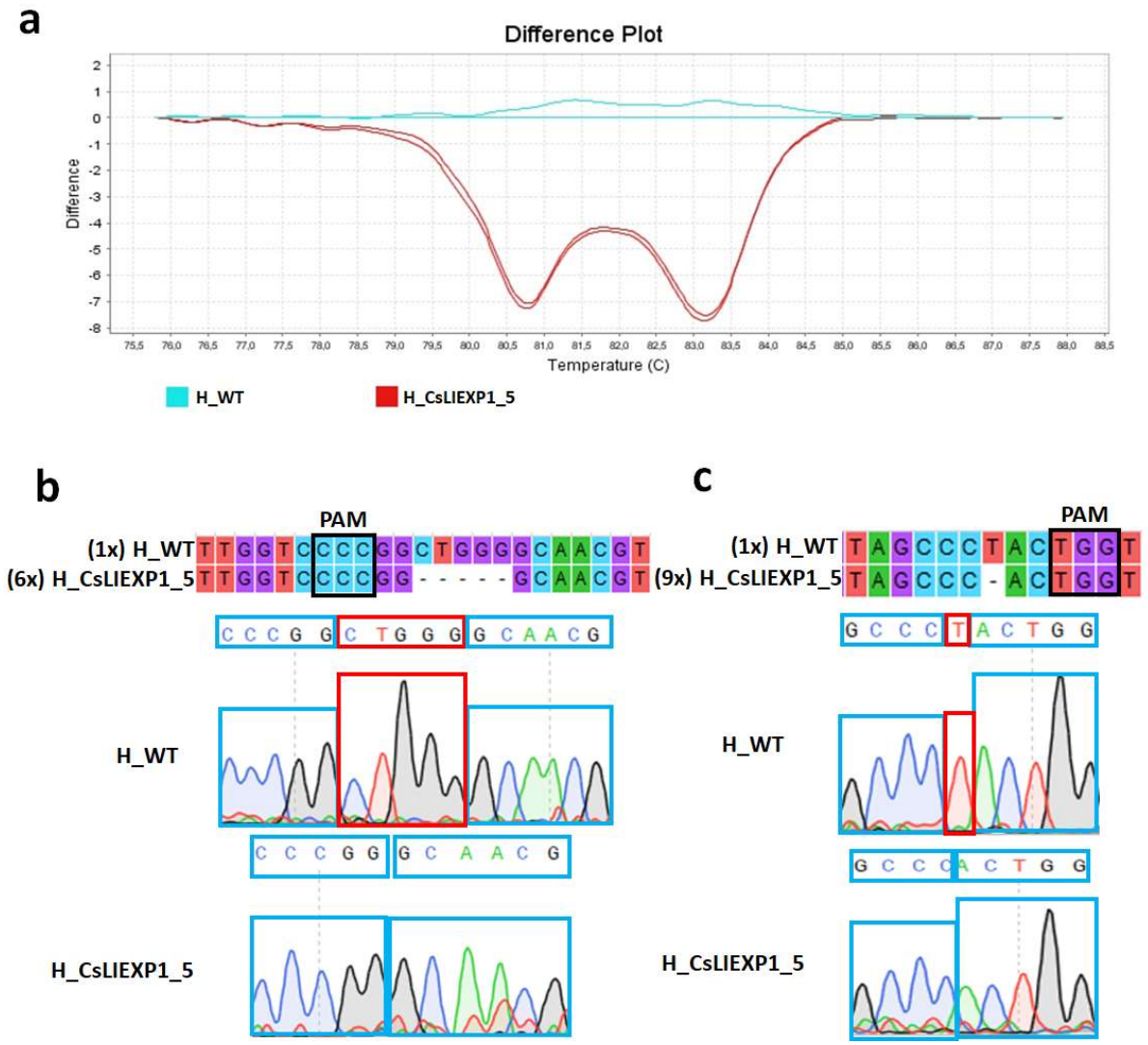

Figure 6. Identification of 93.75% of edited *CsLIEXP1* sequences. (a) HRMA of edited sample (H_CsLIEXP1_2) compared to wild type (H_WT). (b) Sequencing of *CsLIEXP1* target sequencing for CsLIEXP1-gRNA1. (c) Sequencing of *CsLIEXP1* target sequencing for CsLIEXP1-gRNA3. On the top it is demonstrated sequence alignment. On the bottom, representative electropherograms for each sample. The numbers sequenced minipreps are demonstrated left side of each sample. In electropherograms, the up and downstream sequences of edited regions are represented in blue, and the deleted sequences were represented in red.

Frame shift generated by Cas9 drives premature stop codon

The edited plants presented two different frameshifts in *CsLIEXP1* (Figures 5 and 6). We applied these sequences to MEGA7 software (Kumar et al., 2016) for translation of sequences. Premature stop codon was obtained in both amino acid sequences from edited sequences (Figure 7). Deletion of 5 nucleotides in *CsLIEXP1* induced a shift of an alanine by a

glycine at 47th position, which altered the followed amino acids and generate a stop codon at 52nd position (Figure 7). In the sequence with a thymine deleted, a threonine was replaced by a leucine at the 56th position, and the next amino acids were completely different from the wild type sequence which promoted a stop codon at the 79th position (Figure 7). Thus, these results demonstrated that the plants translate a trunked protein, because the two domains present in CsLIEXP1 were not translated, and consequently these proteins were not functional.

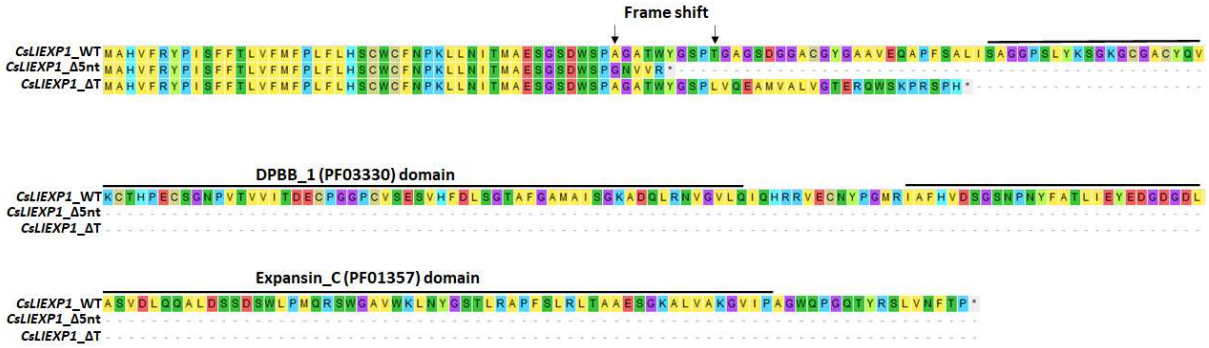

Figure 7. Alignment of wild type and edited CsLIEXP1 amino acid sequences. The predicted amino acid sequences from edited plants were aligned with wild type sequence. The frameshift position created by Cas9 is indicated at the top of figure. Premature stop codon was obtained for both edited sequences. The edited sequences do not have the two expansin domains predicted by Pfam.

CsLIEXP1 edited plant were more resistant to citrus canker

Once we obtained *CsLIEXP1*-edited plant, this plant was challenged with *Xcc*. Young leaves from H_CsLIEXP1_1 and H_WT were infiltrated with *Xcc* and evaluated 14 days after inoculation. Citrus canker symptoms were more evident in H_WT than H_CsLIEXP1_1 in all biological replicates (Figure 8a). In H_CsLIEXP1_1 the symptoms were more restricted to inoculation point (Figure 8a). As we are using *Xcc* constitutively expressing GFP (*Xcc*-GFP) we examined the inoculated plants in GFP filter. Corroborating with visual symptoms, the colonization of *Xcc*-GFP was higher in H_WT plants than H_CsLIEXP1_1 which the colonization was concentrated to inoculation point (Figure 8a). The infiltrated areas were isolated for CFU counting. The average of CFU was performed and the *Xcc*-GFP population was almost 10-fold higher in H_WT than H_CsLIEXP1_4 (Figure 8b). These results demonstrated that *CsLIEXP1* contribute for citrus canker symptoms development. In addition, these findings indicated that even with 43.75% of *CsLIEXP1* edited sequences, the citrus canker symptoms were reduced, which suggest that higher levels of *CsLIEXP1* editing could increase resistance to *Xcc*.

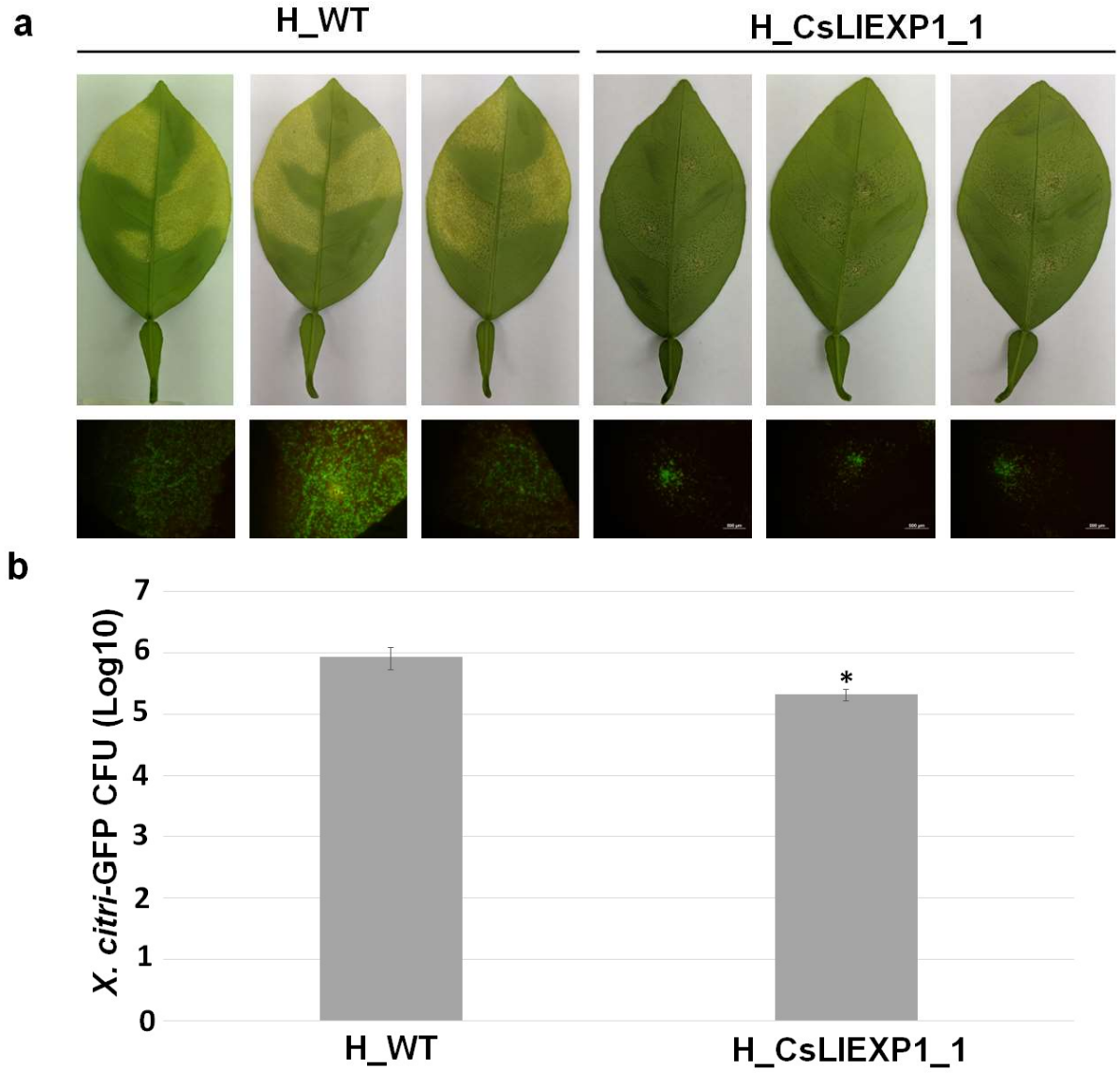

Figure 8. Evaluation of citrus canker resistance. (a) Assessment of citrus canker symptoms 14 DAI in detached leaves inoculated with *Xcc*-GFP. On the top are the representative leaves of wild type (H_WT) and edited (H_CsLIEXP1_1) plants. On the bottom, representative GFP-micrography of inoculated leaves. The green spots indicated *Xcc*-GFP. (b) Average of CFU counting of *Xcc*-GFP isolated from wild type and *CsLIEXP1*-edited plants. CFU from the three replicates were used to perform Student-t test to compare the statistic differences. *: $p < 0.05$.

4.4. Discussion

In this study, HRMA was demonstrated as a powerful method for screening of edited plants and *Xcc*-resistant *CsLIEXP1*-edited sweet orange plants were obtained. First, to obtain these plants CRISPR/Cas9 vector using pDIRECT22C was constructed (Čermák et al., 2017). Genotyping of *CsLIEXP1* first exon was performed to identify conserved regions and

design gRNA in these positions. The sequence was conserved in sweet orange variety Hamlin, confirmed by 30 individual sequences. Based on these sequences, three sgRNAs were designed with 78 nucleotides of distance. Twenty-two transgenic plants were obtained transformed with this construction.

No restriction enzymes sites were identified closed to PAM sequences in *CsLIEXP1* first exon, thus we search for an alternative method for screening of edited plants. HRMA was initially reported as a method for genotyping (Chagné et al., 2008; Wu et al., 2008) and recently used for *LOB1* genotyping in citrus (Peng et al., 2017). In *Arabidopsis thaliana* and *Nicotiana tabacum*, HRMA was described as a rapid and efficient method for screening of edited plants (Chen et al., 2018; Denbow et al., 2017). High accuracy of HRMA was demonstrated which sensed even 10% of edited sequences. Similar results were demonstrated with sequences from *A. thaliana* and *N. tabacum*, which HRMA sense 5% and approximately 3.5%, respectively, of edited sequences (Chen et al., 2018; Denbow et al., 2017). In our experiments, the software identified new variant only with 50% or more of edited sequences. Probably, this is the threshold of software, which was initially designed for genotyping (Applied Biosystems), where normally the samples have 50% of each allele or 100% of one allele. Based on these results, plants with high proportion of edited cells could be selected easily. The HRMA was validated by one sample screened and confirmed by Sanger sequencing. The same variant was identified for the sample and wild type. The sample was confirmed by Sanger sequencing which demonstrated edited sequence lower than 50%. We obtained another sample with 93.75% of *CsLIEXP1* editing and was identified as different variant compared to wild type in HRMA, validating our data, however the shoot did not survive the grafting, and because that was not challenge with *Xcc*. Differently from *A. thaliana* and *N. tabacum*, in this study, the HRMA was used to select an edited perennial plant, such as *C. sinensis*. Transgenic plants were obtained expressing Cas9 and sgRNAs, which facilitated the screening the edited plants. However, in transgene-free approach, which does not have a transgene mark to select the plants by PCR, the screening of edited plants could spend more time, especially for perennial plants which depends on protoplast approaches. In this way, it is necessary to sequence all samples, while using HRMA could make a screening of all samples using only a qPCR technique and select for sequencing only the samples with deformation in melting curve. In addition, HRMA has been widely used for screening of edited sequences in other organism beyond plants such as insects, fish, and stem cells (Carrington et al., 2022; Housden and Perrimon, 2016; Kojin et al., 2021; Pham et al., 2020; Ren et al., 2013).

H_CsLIEXP1_1 line presented 43.75% of edited sequences and drastically reduced the citrus canker symptoms. This result indicated that even a chimeric plant with wild type and edited sequences could be tolerant to *Xcc*. Similar results were obtained in *CsLOB1*-edited lines with 46.91% and 51.12% of gene editing, which were tolerant to *Xcc* (Jia et al., 2017). These frequencies were similar to obtained for *CsLIEXP1*, demonstrating that even the plant was not completely edited it could be tolerant to *Xcc*. Other studies demonstrated that plants with higher levels of *CsLOB1* edited sequences were resistant to *Xcc* (Jia et al., 2017; Jia et al., 2022a; Jia et al., 2022b; Jia and Wang, 2020; Peng et al., 2017). Differently from citrus, rice is a model plant and homozygous edited lines for *OsSWEET11*, *OsSWEET13* and *OsSWEET14* S genes were broad-spectrum resistance to *Xanthomonas oryzae* pv. *oryzae* (Oliva et al., 2019; Xu et al., 2019). In citrus, it was generated *CsLOB1*-edited lines with 100% of gene editing, which were homozygous and biallelic (Jia and Wang, 2020). Although the phenotype is more evident in homozygous edited plants, here we demonstrated that even with 43.75% of *CsLIEXP1* edited sequences was enough to obtain *Xcc* tolerance. Although the difference of symptoms in edited and wild type plants, *Xcc* population isolated from edited plant was approximately 10^5 CFU/mL, which indicates a high *Xcc* population. *Xcc*-tolerant transgenic sweet oranges presented similar results, which demonstrated reduction of symptoms but with high *Xcc* population (Caserta, et al., 2014. Nascimento et al., 2022; de Souza-Neto et al., 2022). Sweet orange plants inoculated with *Xcc*-ΔpthA4 were citrus canker symptoms-free and presented high bacterial population (Abe and Benedetti, 2016), demonstrating that *Xcc* can grow in plant independent of symptoms.

In our experiments, the *CsLIEXP1*-edited plant was tolerant to *Xcc* even with *CsLOB1* activated by PthA4. CsLOB1 induces the high expression of *CsLIEXP1* (de Souza-Neto, data not published ; Duan et al., 2018; Zou et al., 2021). The *CsLIEXP1* is an expansin gene, which function is associated with cell wall loosening and cell expansion (Cosgrove, 2015; McQueen-Mason and Cosgrove, 1995). The *CsLIEXP1*-edited plant reduced *Xcc*-induced pustules, probably because the Cas9-induced frameshifts in *CsLIEXP1* sequences and promotes a premature stop codon which generates trunked proteins. These results indicated that less than 50% of CsLIEXP1 trunked proteins drastically reduced the symptoms, which demonstrated that *CsLIEXP1* significantly contributes to induce citrus canker symptoms. In tobacco, the overexpression of an expansin gene enhanced symptoms caused by *Psedomonas syringae* (Chen et al., 2018). In other hand, the silencing of this gene induced tolerance to the same bacteria (Chen et al., 2018). It has been reported the induction of cell wall loosening by expansins as strategy of plant pathogens to promote diseases or increase virulence (Cosgrove,

2017; Narváez-Barragán et al., 2020; Nikolaidis et al., 2014; Rocha et al., 2020). These plant pathogens use expansin genes from their own genomes or obtained from plants by horizontal gene transfer or controlling plant expansin expression by effectors (Cosgrove, 2017; Narváez-Barragán et al., 2020; Nikolaidis et al., 2014; Rocha et al., 2020; de Souza-Neto, data not published yet). Thus, the editing of expansin genes could be a promise strategy aiming to obtain plant pathogen resistance. In sum, this is the first study that used CRISPR/Cas9 to edit an expansin gene in plant. *CsLIEXP1* play important role to induce citrus canker symptoms and its editing promoted disease resistance. This study open perspectives to use protoplast for gene editing focusing to obtain *Xcc*-resistant transgene-free *CsLIEXP1*-edited plants.

4.5. Experimental procedures

Genotyping of first exon from *CsLIEXP1* gene and sgRNA design

The first exon of *CsLIEXP1* gene (orange1.1t00187, HZAU database) was amplified with specific primers (forward 5' - TATAAAGCTTGCACTACCTATTCATGATTGGC - 3' and reverse 5'- CATCGCTTCCTGCACCAGTAGGG – 3') using Phusion™ High Fidelity – DNA polymerase (Thermo Scientific) from sweet orange variety Hamlin. The PCR product was applied to electrophoresis and the band was purified from gel. The purified amplicon was cloned into pJET1.2 (Thermo Scientific) and transformed in Stellar™ strain of *Escherichia coli* (Takara). Plasmid extraction was performed from PCR positive colonies. These plasmids were confirmed by Sanger sequencing. Thirty sequences were analyzed by Chromas and aligned by MEGA7 software (Kumar et al., 2016). The sequence was applied to CRISPR-P 2.0. software <crispr.hzau.edu.cn> and based on conserved region of first exon from *CsLIEXP1* gene, three sgRNAs were selected.

Construction of CRISPR/Cas9 vector and *Agrobacterium tumefaciens* transformation

The pDIRECT22c was used as CRISPR/Cas9 vector for genetic transformation. The three selected sgRNA, targeting *CsLIEXP1* first exon, were cloned in pDIRECT22c following 3A protocol (Čermák et al., 2017). The final construction (pDIRECT_CsLIEXP1*)* was compounded by CmYLCV promoter expressing 3 sgRNAs with Csy4 sites up e downstream of each one. The pDIRECT_CsLIEXP1 was transformed in *A. tumefaciens* EHA105 strain by electroporation and cultivated in YEP media (1% yeas extract, 1% bactopeptone, 0.5% NaCl) supplemented with rifampicin (50 mg/L) and kanamycin (100 mg/L)

for 72 hours. The colonies were submitted to PCR with specific primers (TC320 5' – CTAGAAGTAGTCAAGGCGGC – 3' and TC089R 5' – GGAACCCTAATTCCCTTATCTGG – 3') (Čermák et al., 2017) to confirm the transformation. pDIRECT_CsLIEXP1 was used as PCR positive control.

Genetic transformation of sweet orange and confirmation by PCR

Seeds of sweet orange (*Citrus sinensis* (L.) Osbeck) variety Hamlin were incubated in half-strength MS medium containing myo-inositol at 100 mg/L, sucrose at 30 g/L and agar at 7 g/L during one month in the dark at 28 °C, for germination and epicotyl development (Caserta et al., 2014). The seedlings were moved for light with photoperiod of 16h, and 5 days after were transformed with *A. tumefaciens* previously transformed with pDIRECT_*CsLIEXP1*.

The bacteria were cultivated overnight in 10 mL of YEP medium with kanamycin (100 mg/L) and rifampicin (50 mg/L) at 28 °C with shaking at 180 rpm. A 3-mL aliquot of the inoculum was added to 50 mL of YEP medium and grown until the OD600 reached 0.4 (Caserta et al., 2014). The culture was centrifuged, and the cell pellet was resuspended in MS liquid medium to an OD600 of 1.0, which inoculated on epicotyl segments.

The explants were infected for 5 minutes with *A. tumefaciens* transformed with pDIRECT_CsLIEXP1 in liquid MS medium containing indole acetic acid at 100 mg/L (Caserta et al., 2014). The explants were dried in sterile filter paper and inoculated in MS media (thiamine at 1 g/L, piridoxine at 5 g/L, nicotinic acid at 5 g/L, glycine at 20 g/L, myo-inositol at 100 mg/L, sucrose at 30 g/L, benzylaminopurine at 1 mg/L, and agar at 6 g/L) for co-cultivation during 3 days at 24 °C and in the dark. Then, the explants were moved to MS media supplemented with kanamycin (100 µg/mL) for selection. The explants were kept in dark until shoots development. After shoots regeneration, they were transferred to light exposition to better development.

DNA from shoots were extracted using CTAB method and used as template to perform PCR reactions with specific primers (TC320 and TC089R) (Čermák et al., 2017). DNA from wild type sweet orange was used as negative control, and pDIRECT_EXP was used as positive control. Positive PCR plants were grafted in Rangpur lime roostock and transferred to greenhouse.

Analysis of HRMA accuracy and screening of edited plants

Sequence from first exon of *CsLIEXP1* gene was used to check the accuracy of HRMA. The wild type and mutant for one thymine (previously obtained in our lab) sequences

were mixed in eleven different ratios (WT:mutant) from 0% until 100 % of muted sequences. These samples were used as template for HRMA reaction. The MeltDoctor™ HRM Master Mix (Thermo Scientific) was used as master mix of reaction following the protocol provided by the company. The primers CsLIEXP1_HRM_F (5' - GTTTCCTCTTTTCCTGCACTCA - 3') and CsLIEXP1_HRM_R (5' – GAAAATACGTACCATCGCTTCCT - 3') were used for the reaction. The reactions were conducted in duplicate in 7500 Fast Real-Time PCR System (Applied Biosystem) with specific calibration for Syto9 dye. The High Resolution Melting Software 2.0.1. (Applied Biosystems) was used to import the results of reaction and analyzed the differences in melt curve. The analyses are compared by the shape of curves, and the difference plot was used as parameter to compare to curves.

DNA previously obtained from transgenic plants were used to perform HRMA as described above using CsLIEXP1_HRM_F and CsLIEXP1_HRM_R primers. The samples were compared to wild type plant, which one was used as negative control. The samples which presented any difference of melting curve shape in difference plot analysis were selected for sequencing as potential edited plant.

Sequencing of *CsLIEXP1* target sequence in transgenic lines

DNA from samples were used to perform PCR with CsLIEXP1_HRM_F and CsLIEXP1_HRM_R primers using Phusion ™ High-Fidelity DNA Polymerase (Thermo Scientific). Products of PCR were applied to electrophoresis and the bands were purified from gel using Wizard® SV Gel and PCR Clean-Up System Protocol (Promega). The samples were cloned into pJET1.2 vector (Thermo Scientific) and transformed in *E. coli* Dh5α strain. The colonies were cultivated overnight in LB media and minipreps were performed. The plasmids were submitted to Sanger sequencing. The quality of sequences was analyzed by SnapGene software. The sequences were aligned by MEGA7 software (Kumar et al., 2016) to identify the indels.

Prediction of proteins from edited plants

The sequences identified in Sanger sequencing from edited plant was translated by MEGA7 software to predict the amino acids sequence. These amino acid sequences were aligned with wild type sequence by MEGA7 software (Kumar et al., 2016) aiming to identify the differences in those sequences.

Challenge of plants against *Xcc*-GFP

Five leaves from wild type and edited plant were infiltrated with *Xcc* 306 strain constitutively expressing GFP (*Xcc*-GFP) (Rigano et al., 2007). Three spots were infiltrated with approximately 100 µL of inoculum 10^4 CFU/mL of *Xcc*-GFP for each leaf. The symptoms were evaluated fourteen days after inoculation (14 DAI). Fluorescence analyses were carried out with inoculated plants using Olympus MVX10 (U-MGFPHQ filter) microscope. After that, leaf discs with 1 cm of diameter of inoculation point were collected and incubated for 15 minutes in phosphate-buffered saline (PBS) in 150 rpm shaking at 28° C. The samples were diluted in PBS and cultivated in NBY media (0.5% peptone, 0.3% meat extract, 0.2% yeast extract, 0.2% K_2HPO_4, 0.05% KH_2PO_4) during two days at 28° C for CFU counting. Student's T-test was used for statical analyses.

4.6. Acknowledgments

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5. Final considerations

In this study, some genes associated to cell proliferation and cell wall remodeling that are potentially regulated by PthA4 and CsLOB1 were identified based on transcriptomic data, *in silico* analysis and relative expression.

The *CsLIEXP1* was more induced than *CsLOB1* in infection with *Xcc*. *N. benthamina* model plant was used as heterologous system to validate experimentally the interaction PthA4-*CsLOB1*-*CsLIEXP1*. *CsLIEXP1* was not regulated by PthA4, only by CsLOB1, which indicates that *CsLIEXP1* is indirectly induced by PthA4. Based on these results and *CsLIEXP1* family function, this gene could contribute to citrus canker development, and therefore it is a new target for genome editing aiming citrus canker resistance.

The *CsLIEXP1* minimum promoter regulated by CsLOB1 was identified and demonstrated that probably CsLOB1 has other unknown binding motifs, which they will be investigated in next studies.

Twenty-two sweet orange transgenic plants expressing Cas9 and the three sgRNA_CsLIEXP1 was obtained. HRMA accuracy test demonstrated that 10% of edited sequences can be detected by the technique, but the software identified new variants only with 50% of edited sequences. Thus, HRMA is a powerful tool for screening of edited plants, able to identify low and high frequencies of edited sequences.

Transgenic samples were applied to HRMA and one plant was selected as putative edited plant. Sequencing of this sample confirmed gene editing. The H_CSLIEXP1_1 presented 43.75% of editing sequences with two types of edited gene: the first one was a deletion of 5 nucleotides and the other one was a deletion of one thymine. These results confirmed *in vivo* the HRMA as method for edited plant screening. Leaves from different branches of this plant was infected with *Xcc*, which reduced bacterial population and citrus canker symptoms development. These results indicated that *CsLIEXP1* contributes to citrus canker development and its editing induced tolerance to this disease. This study open perspectives to apply

CRISPR/Cas technology in protoplasts for *CsLIEXP1* editing aiming obtain transgene-free citrus canker tolerant edited plants.

6. Scientific production during PhD period

Lectures

Lecture given on the topic "Genome editing" in the discipline "In vitro cultivation of plants and Applied Molecular Biology" of the Graduate Program in Plant Production and Bioprocesses, at the Federal University of São Carlos, Araras campus, on May 31 2022, lasting 2 hours.

Lecture given as entitled “Genome editing: Application of CRISPR/Cas9 in plant breeding with a focus on disease resistance” given at the University Extension Course: “Molecular Biology for Plant Disease Control” at the Centro Universitário de Araras “Dr Edmundo Ulson” on May 19, 2022, lasting 2 hours.

Lecture given as entitled “Applications of CRISPR technology in citrus disease control: what has already been done and what are the prospects?” taught in the University Extension Course: "Molecular Biology for Plant Disease Control" at the Centro Universitário de Araras "Dr Edmundo Ulson" on November 21, 2020, lasting of 1 hour.

Lecture given as entitled “Genome editing: Application of CRISPR/Cas9 in plant breeding with a focus on disease resistance” given at the University Extension Course: “Molecular Biology for Plant Disease Control” at the Centro Universitário de Araras “Dr Edmundo Ulson” on October 28, 2021, lasting 2 hours.

Lecture given as entitled “Genome editing: Application of CRISPR/Cas9 in plant breeding with a focus on disease resistance” given at the University Extension Course: “Molecular Biology for Plant Disease Control” at the Centro Universitário de Araras “Dr Edmundo Ulson” on July 22, 2021, lasting 2 hours.

Lecture given as entitled “Applications of CRISPR technology: focus on plant editions aiming at disease resistance” given at the University Extension Course: “Molecular Biology for Plant Disease Control” at Centro Universitário de Araras “Dr Edmundo Ulson” on June 12, 2021, lasting of 1 hour.

Mini course presentation

Mini course given as entitled "High Resolution Melting as a method for screening plants edited via CRISPRCas9" was given at Centro de Citricultura Sylvio Moreira, on March 16 and 17, 2022, lasting 16 hours.

Articles

de Souza-Neto, R. R., Carvalho, I. G. B., Martins, P. M. M., Picchi, S. C., Tomaz, J. P., Caserta, R., Takita, M. A. and de Souza, A. A. (2022) MqsR toxin as a biotechnological tool for plant pathogen bacterial control. Scientific Reports, 12:2794-2805. doi: 10.1038/s41598-022-06690-x.

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Dalio, R. J. D., Magalhães, D. M., Rodrigues, C. M., Arena, G. D., Oliveira, T. S., **Souza-Neto, R. R**., Picchi, S. C., Martins, P. M. M., Santos, P. J. C., Maximo, H. J., Pacheco, I. S., de Souza, A. A. & Machado, M. M. (2017) PAMPs, PRRs, Effectors and R-genes associated with citrus-pathogen interactions. Ann Bot. 119:749-74. doi:10.1093/aob/mcw238.

Presentation in conferences

The work entitled "High Resolution Melting Analysis as a method for screening of edited sweet orange plants by CRISPR/Cas9" was presented in the form of oral presentation in the area of Plant Genetics and Breeding at the IV GBMeeting event held online on January 31st to February 4th 2022.

Reinaldo Rodrigues de Souza-Neto**;** Isis Gabriela Barbosa Carvalho; Fernanda Nogales da Costa Vasconcelos; Doron Teper; Marco Aurelio Takita; Celso Eduardo Benedetti;

Nian Wang; Alessandra Alves de Souza. 66th Brazilian Congress of Genetics held online from September 13 to 16, 2021. The work was presented as a poster in the “Plant Genetics” area entitled “Identification of new susceptibility gene of *Citrus sinensis* to citrus canker.

III GBMeeting: Graduate Meeting in Genetics and Molecular Biology held online from January 25th to 29th, 2021. The work was presented in the oral modality in the area “Plant Genetics” entitled: “Use of CRISPR/Cas9 to edit citrus canker susceptibility genes to obtain commercial sweet orange varieties resistant to *Xanthomonas citri* subsp. *citri*”.

Souza-Neto, R.R, Vasconcelos, F. N. C., Teper, D., Lamichhane, T., Benedetti, C.E., Takita, M. A., Wang, N. and de Souza, A. A. Study presented in poster section named: "New Strategy to Evaluate Putative Susceptibility Genes to Citrus Canker Induced By PthA4". Plant & Animal Genome XXVIII was held from Jan 11th to Jan 15th, 2020 in Town & Country Hotel San Diego, CA, USA.

Reinaldo Rodrigues de Souza-Neto, Celso Eduardo Benedetti, Marco Aurélio Takita, Alessandra Alves de Souza. Identification of putative susceptibility genes to citrus canker mediated by PthA4 effector in different varieties of sweet orange was presented as a poster in VI Xanthomonas Genomics Conference, Halle-Germany, 2018.

Reinaldo Rodrigues de Souza-Neto, Celso Eduardo Benedetti, Marco Aurélio Takita, Alessandra Alves de Souza. The work entitled “Relative expression of putative susceptibility genes modulated by PthA4 effector in sweet orange varieties” was presented as a poster in 2nd São Paulo XanthoMeeting, São Paulo-Brazil, 2018.

Awards

The work "High Resolution Melting Analysis as a method for screening of edited sweet orange plants by CRISPR/Cas9" was awarded in second place in the oral presentation modality in the area of Plant Genetics and Breeding during the IV GBMeeting event held online on January 31st to February 4th 2022.

Top 15 at the 2nd Scientific Day organized by the POST PRIME Relationship Program, Monsanto, Santa Cruz das Palmeiras-SP, 2018.

Project evaluator

Project evaluator in Phase 1 - Innovative Ideas of the Centelha 2 São Paulo Program (FAPESP).

Project evaluator in Phase 2 - Entrepreneurship Project of the Centelha 2 São Paulo Program (FAPESP).

Project evaluator in Phase 3 – Fomentation Projects of the Centelha 2 São Paulo Program (FAPESP).

Courses

Participation in the High Resolution Melting (HRM) Training on the 7500 Fast Real-Time PCR System platform, offered by Thermo Fishers Scientific on July 6, 2021, lasting of 3 hours.

Participation in the Operational Training of the QuantStudio 3 platform, offered by Thermos Fishers Scientific on March 25, 2021, lasting of 4 hours.

Participation in the mini-course “Transforming data into publications” at the III GBMeeting: Postgraduate Meeting in Genetics and Molecular Biology, held online from January 25th to 29th, 2021, lasting of 1 hour.

Participation in the mini-course “Distance Learning: Experiences” at the III GBMeeting: Postgraduate Meeting in Genetics and Molecular Biology, held online from January 25th to 29th, 2021, lasting of 1 hour.

Participation in the mini-course “Methods of communication in English” at the III GBMeeting: Postgraduate Meeting in Genetics and Molecular Biology, held online from January 25th to 29th, 2021, lasting of 1 hour.

Participation in the mini-course "Round Table - Scientific Disclosure" at the III GBMeeting: Postgraduate Meeting in Genetics and Molecular Biology, held online from January 25 to 29, 2021, lasting of 1 hour.

Participation in course entitled “Use of R software for analysis in experimental statistics” held at Instituto Agronômico de Campinas, Campinas-SP, August/September lasting of 20 hours.

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8. Appendix

	SECRETARIA DE AGRICULTURA E ABASTECIMENTO AGÊNCIA PAULISTA DE TECNOLOGIA DO AGRONEGÓCIO INSTITUTO AGRONÔMICO CENTRO AVANÇADO DE PESQUISA E DESENVOLVIMENTO DE CITRICULTURA SYLVIO MOREIRA	

DECLARAÇÃO DE BIOÉTICA

Eu, Raquel Luciana Boscariol Camargo, presidente da CIBio do Centro de Citricultura Sylvio Moreira - IAC, o qual possui CQB no 417/16, declaro que o projeto de tese "**Caracterizaçao de *CsLIEXP1* como um novo gene de suscetibilidade a *Xanthomonas citri* subsp*. citri* e edição gênica por CRISPR/Cas9 para tolerância ao cancro cítrico**", desenvolvido pelo aluno Reinaldo Rodrigues de Souza Neto – RA 158594, no Programa de Pós-graduação em Genética e Biologia Molecular, área de concentração de Genética Vegetal e Melhoramento, da UNICAMP (Universidade Estadual de Campinas), foi realizado dentro das normas de bioética e biossegurança determinadas por essa comissão.

Cordeirópolis, 17 de agosto de 2022.

Dra. Raquel Luciana Boscariol Camargo

Pesquisadora (PqC-VI)

Presidente da CIBio-Citros/ IAC

DECLARAÇÃO DE DIREITOS AUTORAIS

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Tese de Doutorado, intitulada **Characterization of CsLIEXP1 as a new susceptibility gene to Xanthomonas citri subsp. citri and gene editing by CRISPR/Cas9 for citrus canker tolerance,** não infrigem os dispositivos da Lei n. °9.610/98, nem o direito autoral de qualquer editora.

Campinas, 10 de janeiro de 2023.

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