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CROP BREEDING
Article

CsICS gene overexpressed in sweet orange enhanced the resistance to *Candidatus* Liberibacter asiaticus

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ABSTRACT: Huanglongbing (HLB) is a severe disease affecting citrus worldwide, caused by *Candidatus* Liberibacter asiaticus (*C*Las). Transgenic plants overexpressing genes that confer resistance to *C*Las could serve as an effective strategy for controlling HLB. Transgenic citrus plants were previously developed to overexpress the *Citrus sinensis* (*CsICS*) (isochorismate synthase) gene, which is involved in salicylic acid (SA) biosynthesis and may activate systemic acquired resistance (SAR) to bacterial infection. This study evaluated the response of these plants to *C*Las infection. Transgenic sweet orange plants cv. Hamlin were challenged against *C*Las by grafting infected citrus buds, and infection was firstly detected at six months post-inoculation via quantitative polymerase chain reaction. From six to 30 months post-inoculation (mpi), CLas titer, symptom expression, and disease severity were assessed. Gene expression analysis was performed for *C. sinensis*, nonexpressor of PR genes 1 (*CsPR1*) and salicylic acid carboxyl methyltransferase (*CsSAMT*), which are associated with SAR and SA production. Plants of the events *CsICS* 4-4 and 6-11 exhibited infection rates of 7.7 and 6.6%, respectively, while *CsICS* 4-19 showed 28% infection, compared to 64% in wild-type plants. The *CsICS* 4-4 event demonstrated increased resistance and significantly lower bacterial concentrations (7.15×10^3 CLas per gram of tissue) compared to the wild type (1.77×10^6 *C*Las per gram of tissue) at 12 mpi. Notably, *CsICS* 4-4 plants lacked characteristic HLB symptoms. Gene expression analysis confirmed that *CsICS* gene expression was upregulated in transgenic plants regardless *C*Las infection. The transgenic events showed a significant response to *C*Las, with reduced symptom severity and fewer infected plants, highlighting their potential for HLB management.

Key words: huanglongbing, genetic breeding, systemic acquired resistance, isochorismate synthase.

INTRODUCTION

Citrus plants are widely cultivated worldwide, with China, Brazil, and the United States of America being the largest producers. Various diseases negatively impact citrus production, including citrus canker (*Xanthomonas citri* subsp. *citri*), citrus variegated chlorosis (*Xylella fastidiosa* subsp. *pauca*), leprosis (*Cilevirus leprosis*), and huanglongbing (HLB, *Candidatus* Liberibacter spp.) (Wang 2020).

Citrus greening or HLB is the most devastating citrus disease worldwide (Bové 2006), predominantly caused by *Candidatus* Liberibacter asiaticus (*C*Las), a fastidious and Gram-negative bacterium (Merfa et al. 2019). Citrus plants infected with HLB exhibit physiological, anatomical, and morphological changes, including reduced plant size compared to healthy citrus, asymmetrical leaf mottling, leaf and fruit drop, low soluble solid content in juice, and a compromised root system (Bové 2006, Johnson et al. 2014, Nehela and Killiny 2020, Raiol-Junior et al. 2021). Furthermore, CLas is naturally transmitted by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Bové 2006).

The absence of resistant or tolerant citrus genotypes to HLB hampers disease control strategies and hinders genetic improvement efforts, making it a significant challenge to achieve resistance through traditional breeding methods (Boscariol-Camargo et al. 2010, Graça et al. 2016, Dorta et al. 2019). Nevertheless, plant genetic engineering techniques have been explored to develop resistant or tolerant genotypes. In the absence of such genotypes, management strategies are employed to control the disease, including psyllid control through chemical or biological methods, planting in unaffected or low-incidence areas, using healthy seedlings, inspecting and removing infected plants, among other techniques (Pandey and Wang 2019).

The plant immune response begins with the recognition of an invading pathogen, triggering specific defense mechanisms such as pathogen-associated molecular pattern-triggered immunity, effector-triggered immunity, and systemic acquired resistance (SAR). Salicylic acid (SA) is a plant hormone that plays a fundamental role in various physiological responses, including local and systemic plant defense. It is synthesized via the phenylpropanoid pathway or through isochorismate synthase (ICS) in the chloroplast (Chen et al. 2009). The isochorismate pathway is the primary route for SA biosynthesis in plants and is essential for SAR activation in *Arabidopsis thaliana* (Wildermuth et al. 2001, Dempsey et al. 2011). The pathway begins with the ICS enzyme, which catalyzes the conversion of chorismate into isochorismate, which is subsequently converted into SA by isochorismate pyruvate lyase (*PL*) (Darolt et al. 2020). A key component of the SAR system is the S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase (*SAMT*) gene, responsible for converting SA into a volatile signal (MeSA). Nascimento et al. (2022) demonstrated that transgenic sweet orange (*Citrus sinensis*) plants overexpressing the *CsSAMT* gene activated SAR and exhibited resistance to citrus canker. Similarly, Zou et al. (2021) reported that sweet orange plants overexpressing *CsSAMT* showed increased resistance to HLB.

Another key gene involved in SAR activation via SA is the nonexpressor of PR genes 1 (*NPR1*), which responds to increased SA levels by positively regulating SA-responsive genes and triggering SAR (Kumar 2014, Nascimento et al. 2022). Overexpression of NPR1 from *Arabidopsis thaliana (AtNPR1)* and its homolog from *Citrus maxima* (Burm.) Merr. (*CtNH1*) enhanced resistance to citrus canker in "Duncan" grapefruit (*Citrus paradisi* Macfadyen), "Hamlin" sweet orange, and pumelo (*C. maxima*), respectively (Boscariol-Camargo et al. 2016, Chen et al. 2013, Zhang et al. 2010).

To investigate whether SA biosynthesis in *Nicotiana benthamiana* is derived from and dependent on *ICS*, as described for *A. thaliana* (Wildermuth et al. 2001), Catinot et al. (2008) silenced the *ICS* gene in *Nicotiana benthamiana*. When plants were inoculated with *Pseudomonas syringae*, no SA accumulation was observed in *ICS*-silenced plants, confirming the pathway's importance. In citrus, Darolt et al. (2020) reported positive regulation of *ICS*, *SGT1*, and *PAL* genes in plants inoculated with *C*Las, indicating that the plant responds to the bacterium by activating the SA response pathway. The activation of this pathway in response to *C*Las infection may mitigate the physiological and morphological damage caused by the bacterium.

In this study, we evaluated the response of transgenic sweet orange plants overexpressing *CsICS* following *C*Las infection. The hypothesis was that transgenic citrus plants overexpressing genes of SA pathway could impair the *C*Las infection.

MATERIALS AND METHODS

Plant material and confirmation of transgenesis

Transgenic sweet orange (*C. sinensis* L. Osbeck) plants of the "Hamlin" variety overexpressing the *CsICS* gene were previously developed (unpublished data) at the Sylvio Moreira Citrus Center, Instituto Agronômico de Campinas, Cordeirópolis, SP, Brazil, and used in this challenge experiment against *C*Las infection. Transgenic plants were generated using *Agrobacterium tumefaciens*-mediated transformation with the pCambia 2301 vector, which carries the *CsICS* gene under the control of the constitutive Ubi10 (ubiquitin) promoter and the OCS terminator, along with the *uidA* (Gus) reporter gene and the *nptII* (kanamycin resistance) selection gene. Transformation was confirmed through Gus staining, polymerase chain reaction (PCR), and quantitative polymerase chain reaction (qPCR) analyses.

The transgenic plants were propagated by grafting onto Rangpur lime (*Citrus limonia* Osbeck) rootstock and maintained under greenhouse conditions. Eighteen replicates of each *CsICS* transgenic event, along with non-transgenic "Hamlin"

wild-type plants, were produced, totaling 72 plants. To confirm the transgenesis in the propagated transgenic plants, a histochemical Gus assay was performed. Leaf sections were immersed in *X-GLUC* (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) solution and incubated overnight at 37°C in the dark (Lacorte and Romano 1998), followed by washing with a 3:1 acetic acid/ethanol solution.

*C*Las transmission and infection

*C*Las was transmitted to the test plants (15 replicates of each event) through grafting by budding from infected citrus plants. The sweet orange plants serving as *C*Las sources exhibited typical HLB symptoms and were tested for the presence of *C*Las via PCR before bud collection. For *C*Las transmission, two approximately 2-cm-long buds from infected source plants were grafted onto the main stem of the rootstock per plant. Wild-type (non-infected control) plants received buds from healthy donor plants under the same conditions.

*C*Las infection was confirmed by PCR at four months post-infection (mpi) using A2/J5 primers, which partially amplify the *rplKAJLrpoBC* gene of *C*Las, as described by Hocquellet et al. (1999). Leaf samples (300 mg of petiole and midrib tissue) were collected for DNA extraction using the TissueLyser II apparatus (Qiagen, Hilden, Germany). Samples were homogenized with two 5-mm-diameter beads in 2-mL microtubes at 30 Hz/s for 3 minutes. DNA extraction followed the cetyltrimethylammonium Bromide (CTAB) method (Murray and Thompson 1980). The PCR assay included 10 µM of A2/J5 primers, 2.65 µL of GoTaq buffer (Promega Corporation, Madison, WI, United States of America), 8.5 µL of autoclaved deionized water, and 1 µL of total plant DNA, for a final reaction volume of 13 µL. PCR amplifications followed: 94°C for 3 minutes, 94°C for 30 seconds, 55°C for 20 seconds, and 72°C for 45 seconds (repeated 35 times), with the final extension at 72°C for 3 minutes.

Evaluation of symptoms

HLB symptom assessments were conducted monthly after the first plant exhibited disease-associated symptoms, as described by Bové (2006). In cases in which symptoms were absent, asymptomatic leaves were chosen instead. All selected leaves were marked for continuous symptom monitoring over 12 months, with additional assessments conducted at 30 mpi.

Quantification of CLas using quantitative polymerase chain reaction

Bacterial titer quantification was performed at 6, 8, 12, and 30 mpi. At each sampling time point, two leaves were collected per plant, and 300 mg of tissue (petiole and part of the midrib) were used for DNA extraction. The extraction was carried out using the TissueLyser II apparatus (Qiagen, Hilden, Germany) with two 5-mm-diameter beads in 2-mL microtubes, at the frequency of 30 Hz/s for 3 minutes. DNA extraction followed the CTAB method (Murray and Thompson 1980), and DNA quantification was performed using the NanoDrop ND-8000 spectrophotometer (Thermo Scientific, Waltham, MA, United States of America), adjusting the total DNA concentration of each sample to 100 ng/µL.

*C*Las quantification was conducted using qPCR with the TaqMan system on the 7500 Fast instrument (ABI PRISM 7500 Fast Sequence Detection System, Applied Biosystems, Thermo Fisher Scientific). Specific primers and probes targeting the 'elongation factor Ts' (*efTU*) gene were used as described by Lin et al. (2010). Additionally, the glutathione S-transferase (*GAPDH*) gene was included in the same reaction to monitor DNA quality and concentration (Boava et al. 2015). The reactions consisted of 0.4 µM primers and 0.2 µM of the *C*Las probe, 0.048 µM primers and 0.024 µM of the *GAPDH* probe, 6.75 µL of TaqMan master mix (PCR Biosystems Ltd, London, United Kingdom), 1.95 µL of dH2O, and 100 ng of DNA (final volume of 13.5 µL). The amplifications followed standard qPCR conditions (95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute).

The *C*Las concentration in the plants was determined according to Wang et al. (2006). A linear regression equation (y = -2.3385x + 36.258, R^2 = 0.9924) was generated by plotting cycle threshold (Ct) values obtained from the qPCR technique against known concentrations of plasmid DNA containing the cloned insert of the *C*Las *efTU* gene used as the

target. Assuming a single copy of the target gene in the *C*Las genome, this regression equation was used to extrapolate the number of *C*Las cells from the Ct values obtained for each sample (Coletta-Filho et al. 2010).

Gene expression analysis

Gene expression of the CsICS, PR1, and SAMT genes were performed using the reverse transcription qPCR (RT-qPCR) technique on leaves from plants in which the presence of *C*Las was confirmed, as well as on non-inoculated plants. The following primer sequences for RT-qPCR were used: *CsICS* (F: 5′GTTCTGTCTTCAG CCTCCTGAT 3′and R: 5′AAGCAGTTGTTCCACCTCTACC 3′); SAMT (F: 5′GGAGGTGGTTCAAGTGCTTC 3′ and R:5′TGGCTTTGCAATGGATATGA); and PR1 (F:5′ATGCGCAAAATTATGCTTCC3′ and R:5′TCATCGACCCACATCTCAAC). RNA extractions were carried out from samples that tested positive for *C*Las and from wild type after the sampling period for CLas quantification, specifically at 12 mpi.

RNA extraction from the leaves was performed using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Subsequently, sample quantification and cDNA synthesis were carried out using the Revertaid H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Waltham, Massachusetts, United States of America) from the extracted RNA treated with DNase. The RNA was treated using the DNase I, RNase-free kit (Thermo Scientific, Waltham, Massachusetts, United States of America), and then cDNA was synthesized from a reaction containing Oligo(dT), 5x reaction buffer, Ribolock RNase inhibitor, 10 mM dNTP mix, and RevertAid H minus M-MuLV reverse transcriptase. qRT-PCR analyses were performed on the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, United States of America) using the SYBR-Green detection system (Wittwer et al. 1997). Primers were designed using the Primer3Plus program (Untergasser et al. 2007). Amplifications were performed in triplicate, always including non-cDNA control to detect possible contaminations. The reference genes used for sample normalization were *EF-1α* and glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPC2*), as described by Mafra et al. (2012). These genes were used to standardize the samples in case of potential differences in cDNA concentration.

Amplifications were carried out in a final volume of 10 µL, comprising 5 µL of GoTaq qPCR Master Mix (Promega, Madison, WI, United States of America), 1 µL of concentrated cDNA obtained from 1 µg of purified RNA, and 200 nM of each primer pair. Reactions were performed with a cycling protocol of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The expression level of the target gene was calculated using Eq. 1, following Livak and Schmittgen's (2001) method.

$$2^{-\Delta\Delta Ct} = \Delta Ct\ (\text{sample}) - \Delta Ct\ (\text{calibrator}) \quad (1)$$

Statistical analyses

The experiment was carried out in a completely randomized design. The statistical analyses for mean comparisons were performed using analysis of variance with Student's t-test and Tukey's test, both with the significance level of $p < 0.05$. The software SAMS-AGRI (http://www.alice.cnptia.embrapa.br/alice/handle/doc/512901) and Excel were used in these analyses.

RESULTS AND DISCUSSION

At the end of 30 months of evaluation, the percentage of genetically modified plants testing positive for *C*Las ranged from 6.6 to 28.6%. However, it is important to highlight the low infection rate obtained in the *CsICS* 6-11 and *CsICS* 4-4 events, in which only 6.6 and 7.7% of inoculated plants tested positive (Fig. 1). The first confirmation of *C*Las presence occurred at six months post-inoculation (mpi) in plants of the *CsICS* 4-19 event and in wild-type plants. Notably, the *CsICS* 4-4 event stood out in this study, as only one plant tested positive throughout 30 months evaluation period, with the bacterium being detected only at 12 mpi (Fig. 1).

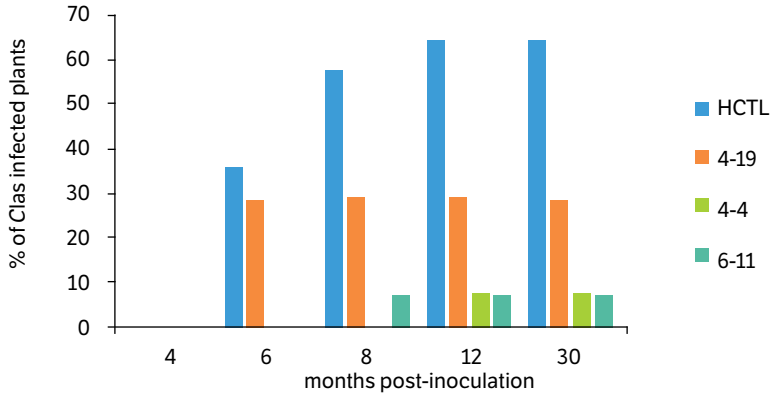


Figure 1. Number of infected plants (%) after four, six, eight, 12 and 30 months after *Candidatus* Liberibacter asiaticus (*C*Las) inoculation in *Citrus sinensis* (*CsICS*) transgenic plants (lines 4-19, 4-4, and 6-11) and the wild type (HCTL).

CLas was firstly detectable at 6 mpi for both *CsICS* 4-19 transgenic line and wild-type (HCTL) plants, with bacterial concentrations of 2.49×10^8 and 4.79×10^6 copy number/gram (CN/g) of tissue, respectively (Table 1). In contrast, plants of the *CsICS* 4-4 and *CsICS* 6-11 events were firstly infected only at 8 and 12 mpi, with bacterial concentrations at 7.15×103 and 4.74×10^5 CN/g, respectively. *CsICS* 4-4 hosted approximately 1,000 times less *C*Las compared to wild-type plants (1.77×10^6 CN/g of tissue) 12 mpi. Compared to the other transgenic events such as *CsICS* 4-19 and CsICS 6-11, *CsICS* 4-4 also exhibited the lowest *C*Las concentration in that period (Table 1).

Table 1. Concentration of *Candidatus* Liberibacter asiaticus (*C*Las) in transgenic (*CsICS*) and wild-type "Hamlin" sweet orange plants.

Event	Months post inoculation							
	6		8		12		30	
	Ct[a]	CLas CN/g	Ct	CLas CN/g	Ct	CLas CN/g	Ct	CLas CN/g
4-4	und[ns]	----	und[ns]	-----	31.36*	7.15×10^3	18.08[ns]	3.41E + 09
4-19	20.74*	2.49×10^8	24.78[ns]	4.66×10^6	24.94[ns]	3.98×10^6	18.57[ns]	2.13E + 09
6-11	und[ns]	----	24.36[ns]	7.05×10^6	27.1[ns]	4.74×10^5	24.51[ns]	6.07E + 06
Wild type	27.45	4.79×10^6	27.04	5.03×10^5	25.76	1.77×10^6	21.33	1.39E + 08

[a]The Ct values refer to the average of only the infected plants. Plants with indeterminate Ct (absence of infection) were not considered in the analyses; CN: copy number of the target gene '*elongation factor Ts*' of CLas per gram of plant tissue; *significant difference ($p \leq 0.05$) when the values were compared to those obtained for the wild-type; *non-significant differences when the values were compared to those obtained for the wild-type.

After 30 months of inoculation, bacterial concentrations were high in all infected plants, ranging from 6.07×10^6 to 3.41×10^9 CN/g of tissue. However, the number of infected plants was still significantly lower in plants from both transgenic events *CsICS* 6-11 and *CsICS* 4-4 compared to the wild-type plants. Only one *C*Las-positive plant was able to be detected for each event over 30 months of experimentation, compared to nine *C*Las-detected wild-type plants. The reduced number of infected plants in these transgenic events, along with the lower severity of symptom, suggests that the *ICS* gene plays a key role in the plant's response to *C*Las infection and colonization.

The first typical symptoms of HLB were observed at 8 mpi in the *CsICS* 4-19, *CsICS* 6-11, and wild-type plants. At this time, Ct values ranged between 24 and 27, with a bacterial population of approximately 10^5 to 10^6 cells per gram of tissue (based on the number of copies of the *efTU* gene) in both transgenic and non-transgenic plants. At 12 mpi, *CsICS* 4-4 plants were asymptomatic for HLB. This event maintained a higher number of green leaves, higher plant height compared to the wild-type plants, increased branching, and enhanced overall vigor. These observations suggested that the *CsICS* 4-4 transgenic line impairs *C*Las infection, probably due to the protective action of the ICS gene (Fig.2).

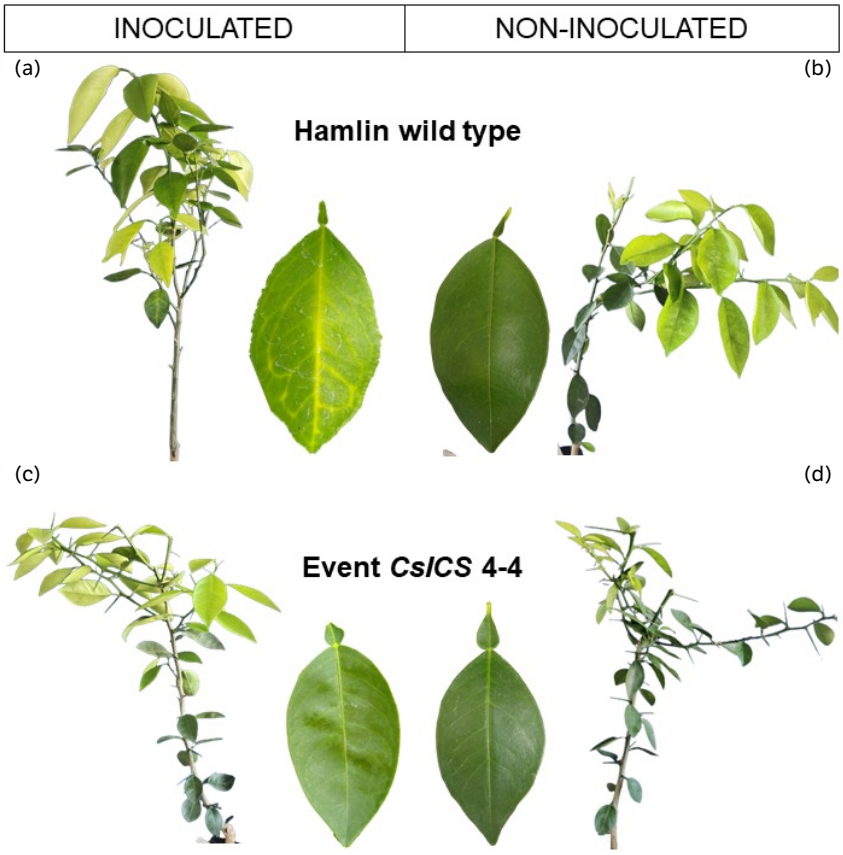


Figure 2. Phenotypic evaluation of transgenic plant 4-4 and the wild-type "Hamlin" plant. (a and b) Wild-type events: (a) the plant infected with *Candidatus* Liberibacter asiaticus (*C*Las); (b) the non-inoculated plant. (c and d) Citrus sinensis (*CsICS*) 4-4 event: (c) the infected plant; (d) the non-inoculated plant.

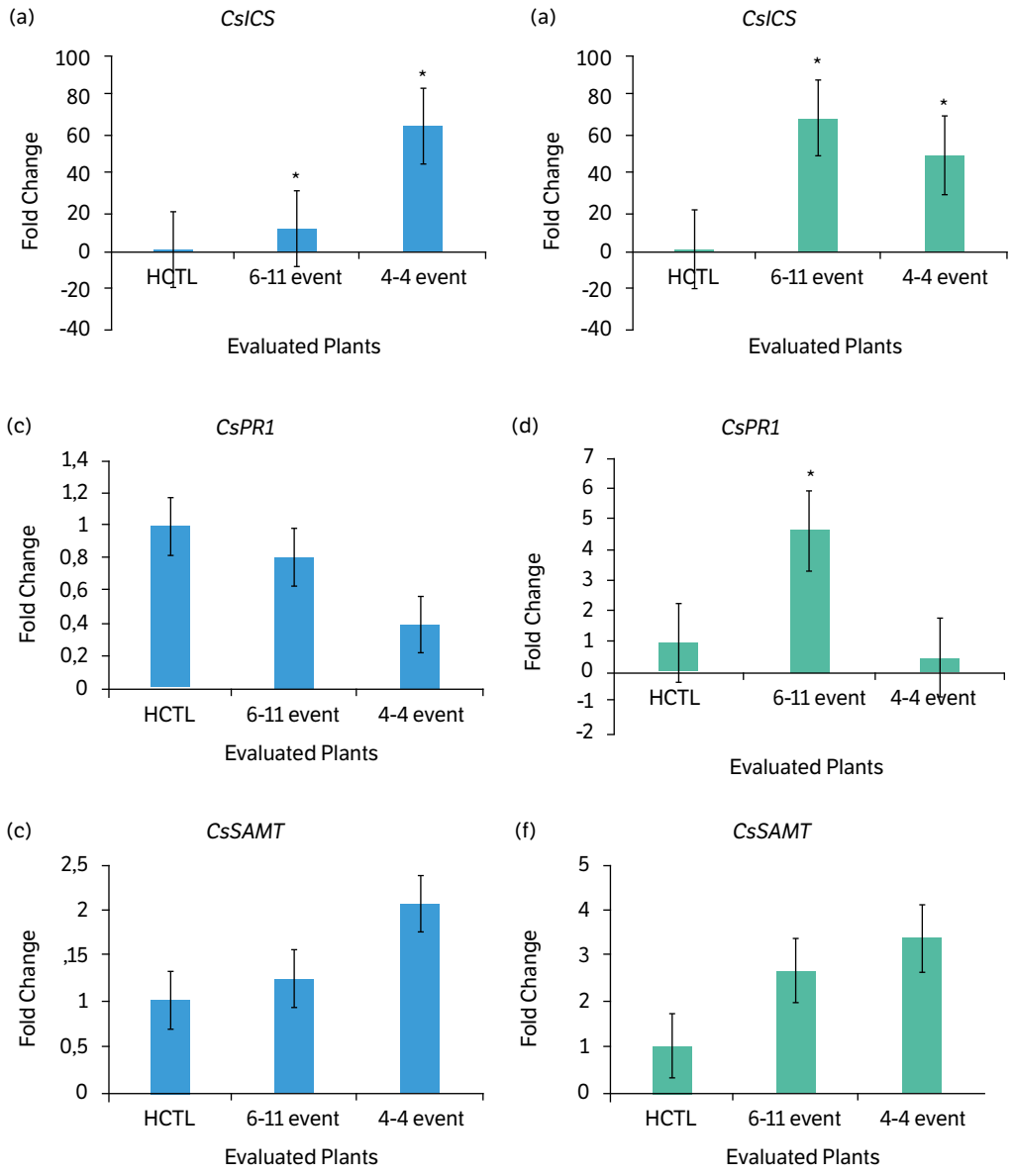
The expression levels of the *CsICS* gene were higher in the transgenic plants (6-11 and 4-4 events), regardless they were inoculated with *C*Las or not, showing a significant difference compared to the wild type (Figs. 3a and 3b).

On the other hand, in the presence of the pathogen, the up-regulation of *ICS* gene was significantly increased, suggesting an accelerated SAR in the plants. It is important to contextualize that the constitutive activation of *CsICS* gene may boost the defense system of plants against the *C*Las infection (Fig. 1).

For the expression levels of the *CsPR1* gene associated with plant defense response through the SA pathway, no significant differences were observed in non-inoculated plants with *C*Las when compared to the wild type (Fig. 3c). However, in plants inoculated with *C*Las, a significant difference was observed for event 6-11 compared to the wild type (Fig. 3d), indicating that this gene is also positively regulated by bacterial infection, like *CsICS*. Regarding the expression analysis of the *CsSAMT* gene, also associated with the plant defense response through the SA pathway, in non-inoculated plants there were no significant differences between wild-type and transgenic lines, but with increased of gene expression in the engineered plants, mainly for the event 4-4 (Fig. 3e). The same trend of expression for the *CsSAMT* gene was observed when *C*Las was present in the plants, although no statistical significance was observed, despite the increase of two or three times of expression level compared to HCTL (Fig. 3f).

Among several variables impacting the significant differences for the *PR1* and *SAMT* genes expression, the sampling at 12 mpi may have been too late. These genes are known to be involved in the early stages of SA pathway activation (Boscariol-Camargo et al. 2016, Zou et al. 2021). The *ICS* plays a crucial role in the biosynthesis of SA, an important signaling molecule in plants. Studies have shown that SA is essential for activating the plant's defense system against biotrophic and semi-biotrophic pathogens, as plants with deficiencies in SA biosynthesis are severely limited in their resistance to these types of pathogens (Fu and Dong 2013, Qi et al. 2018). One of the main effects of SA in plant defense

is the induction of the expression of pathogenesis-related (*PR*) genes, which encode proteins with antimicrobial activities. Among these genes, *PR1*, *PR2*, and *PR5* are strongly induced during infection by biotrophic and semi-biotrophic pathogens (Qi et al. 2018). The exogenous application of SA positively regulates the plant's defense against pathogens, leading to a reduction in bacterial pathogen titers and the expression of virulence factors (Bandara et al. 2006, Lu 2009, Joshi et al. 2016, Qi et al. 2018).



*Significant differences ($p \leq 0.05$) when the values were compared to those obtained for the wild type ("Hamlin").

Figure 3. Expression of the *Citrus sinensis* (*CsICS*), nonexpressor of PR genes 1 (*CsPR1*), and salicylic acid carboxyl methyltransferase (*CsSAMT*) genes. (a) The expression of the genes in non-inoculated plants with *C*Las (black color). (b) The expression of the genes in plants inoculated with *C*Las (blue color). (c) The expression of the genes in non-inoculated plants with CLas (black color). (d) The expression of the genes in plants inoculated with *C*Las (blue color). (e) The expression of the genes in non-inoculated plants with *C*Las (black color). (f) The expression of the genes in plants inoculated with *C*Las (blue color). The wild type (HCTL), 6-11 and 4-4 are transgenic events.

It is well established that the *CsICS* gene plays a key role in the SA biosynthesis pathway, a crucial hormone for the plant's defense response, particularly in SAR against pathogen infections (Darolt et al. 2020). The results from the current study on the transgenic events *CsICS* 4-4 and *CsICS* 6-11 corroborate findings by Zou et al. (2021), who observed that plants

transformed with the *CsSAMT1* gene (another gene in the SA pathway) did not exhibit clear symptoms of HLB. Consistent with the beneficial effects of SA in mitigating HLB, Li et al. (2021) demonstrated a reduction in symptoms in *C*Las-infected plants when treated with SA compared to untreated plants.

In this study, transgenic plants overexpressing *CsICS* exhibited enhanced resistance to *C*Las, evidenced by reduced HLB symptoms and a lower number of infected plants (Table 1, Fig. 1). Similarly, Zou et al. (2021) reported that overexpression of *CsSAMT1* in *C. sinensis* enhanced tolerance to HLB, suggesting the involvement of the SAR pathway. These resistance phenotypes likely result from the activation of SAR through SA signaling and MeSA production. The exogenous application of SA and SAR inducers has been shown to confer resistance to *Xanthomonas citri* (Graham and Myers 2011, Wang and Liu 2012).

Notably, plants of the *CsICS* event 4-4 did not show typical disease symptoms (Fig. 2). SA is an essential plant defense hormone, promoting immunity in response to both biotic and abiotic stresses (Koo et al. 2020, Peng et al. 2021, Gao et al. 2023). Additionally, previous studies have shown that SA can inhibit auxin signaling pathways, contributing to the plant's defense mechanisms (Wang et al. 2007, Kazan and Manners 2009, Gao et al. 2023). In tolerant "Shatian" pomelo, the significant upregulation of the SA pathway may lead to the global suppression of auxin signaling, suggesting that SA-mediated plant defense plays a critical role in the tolerance of "Shatian" pomelo during CLas infection (Gao et al. 2023). Given the crucial role of SA in plant defense, modulating its levels could enhance resistance to phytopathogens.

CONCLUSION

The results presented here suggested that overexpression of the *CsICS* gene in transgenic events 4-4 and 6-11 strongly reduced bacterial infection, potentially activating the plant defense response faster than in the wild-type plant. Furthermore, when infection occurred in only one plant from each event, the onset of HLB symptoms was delayed. This highlights the potential of using the *CsICS* gene as a promising approach to enhance HLB resistance. These events (*CsICS* 4-4 and 6-11) should be tested under field conditions to evaluate their effectiveness in real-world environments.

CONFLICT OF INTEREST

Nothing to declare.

AUTHORS' CONTRIBUTION

Conceptualization: Boscariol-Camargo, R. L. and Freitas-Astúa, J.; **Investigation:** Boscariol-Camargo, R. L., Rampim, B. T. and Coletta-Filho, H. D.; **Formal Analysis:** Rampim, B. T. and Coletta-Filho, H. D.; **Methodology:** Rampim, B. T. and Coletta-Filho, H. D.; **Funding Acquisition:** Boscariol-Camargo, R. L. and Coletta-Filho, H. D.; **Writing – Review and Editing:** Rampim, B. T., Freitas-Astúa, J., Boscariol-Camargo, R. L. and Coletta-Filho, H. D.; **Supervision:** Boscariol-Camargo, R. L. and Coletta-Filho, H. D.; **Project Administration:** Boscariol-Camargo, R. L. and Coletta-Filho, H. D.; **Final approval:** Boscariol-Camargo, R. L.

DATA AVAILABILITY STATEMENT

All datasets were generated and analyzed in the current study.

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