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Research Article

Overexpression of the Arabidopsis NPR1 protein in citrus confers tolerance to Huanglongbing

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Abstract

Huanglongbing (HLB), also known as citrus greening, is one of the most destructive diseases of citrus worldwide. It is caused by unculturable phloem-limited bacteria that belong to the *Candidatus* Liberibacter genus including *Ca.* L. asiaticus (*C*Las), *Ca*. L. africanus, and *Ca.* L. americanus. Currently, there is no effective control strategy for HLB and no known cure for the disease. We have previously generated transgenic 'Duncan' grapefruit and 'Hamlin' sweet orange expressing the *Arabidopsis thaliana NPR1* (*AtNPR1*) gene, which encodes a master regulator of systemic acquired resistance. Characterization of the transgenic lines indicated that overexpression of *AtNPR1* confers resistance to citrus canker, another serious bacterial disease in citrus. In this study, we intensively screened these transgenic lines for resistance or tolerance to HLB under greenhouse conditions. Three independent transgenic lines (one line in the 'Duncan' grapefruit background and two lines in the 'Hamlin' sweet orange background) only occasionally displayed mild HLB symptoms in the presence of *C*Las and have kept growing normally over a period of nine years. Significantly, all vegetatively propagated progeny plants of these lines have retained the same levels of HLB tolerance. Furthermore, immunoblot analysis revealed that the three transgenic lines accumulate high levels of AtNPR1 protein. These results, together with the previous finding that the same three transgenic lines are resistant to citrus canker, demonstrate that overexpression of *AtNPR1* in citrus is able to provide robust tolerance to HLB.

Keywords: Huanglongbing, the Arabidopsis *NPR1* gene, *Candidatus* Liberibacter asiaticus, tolerance, transgenic plants

Introduction

Huanglongbing (HLB), also known as citrus greening, is one of the world's most devastating citrus diseases. HLB infection often leads to drastic reduction in the quantity and quality of citrus fruits and eventually renders the infected trees useless (Gottwald 2010; Wang and Trivedi 2013). In areas affected by HLB, the average productive lifespan of citrus trees drops dramatically from 50 or more years to 15 or less. HLB is causing significant damage in citrus production areas. For instance, since HLB was first identified in 2005, total citrus acreage in Florida has shrunk by more than 50%, an unprecedented contraction of the Florida citrus industry.

HLB is caused by the so far unculturable phloem-limited bacteria that belong to the *Candidatus* Liberibacter genus (Jagoueix et al. 1994; Garnier et al. 2000). Three species of the genus, *Ca.* L. asiaticus (*C*Las), *Ca.* L. africanus, and *Ca.* L. americanus, have been associated with HLB (Bove 2006). The bacterial pathogens are transmitted by the Asian citrus psyllid *Diaphorina citri* or the African citrus psyllid *Trioza erytreae* (Aubert 1987). Psyllids move quickly when disturbed and can fly at least 1.25 miles (Moffis et al. 2016). They can also be carried over long distances by wind, which significantly accelerates the spread of HLB. Although pesticide treatments for the vector could help slow the spread of the disease, the treatments not only greatly increase production cost but also disrupt integrated pest management programs for other citrus pests. Unfortunately, there is currently no effective control measure for HLB and no known cure for the disease.

It has become clear that the best long-term solution for management of HLB is to create resistant or tolerant cultivars of important citrus species. However, field and greenhouse studies have shown that almost all commercial citrus cultivars are susceptible to HLB (Folimonova et al. 2009; Miles et al. 2017). In a recently published long-term field evaluation (Ramadugu et al. 2016), resistance/tolerance was observed in some citrus relatives such as the Australian genera *Eremocitrus* and *Microcitrus*, which fortunately are sexually compatible with citrus. However, introduction of the HLB resistance/tolerance into citrus by breeding is expected to be difficult, because citrus species are highly

heterozygous, polygenic plants with a long juvenile period, and the genetic composition of the citrus relatives is expected to interfere with the expression of optimum traits related to fruit quality and production. These together make the product of conventional crossbreeding too late to save the threatened industries.

It has been well documented that transgenic approaches are able to incorporate resistance into crops with negligible effects on the expression of optimum varietal traits. A large number of genes have been tested for their potential to improve disease resistance in crop plants. Among these genes, the *Arabidopsis thaliana NPR1* (*AtNPR1*) is perhaps the most extensively studied example. *AtNPR1* encodes a master positive regulator of systemic acquired resistance (SAR). Arabidopsis *npr1* mutant plants are defective in SAR induction and highly susceptible to pathogen infections (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). On the other hand, it has been shown that transgenic Arabidopsis plants overaccumulating the AtNPR1 protein exhibit increased resistance to bacterial and oomycete pathogens (Cao et al. 1998; Friedrich et al. 2001). Moreover, *AtNPR1* and its orthologs have been transformed into diverse crop plants to test their effects on disease resistance (Silva et al. 2017). Successful examples have been reported in many crops including rice, wheat, rapeseed, tomato, tobacco, apple, citrus, and strawberry (Silverman et al. 1995; Cao et al. 1998; Chern et al. 2001; Friedrich et al. 2001; Lin et al. 2004; Chern et al. 2005; Makandar et al. 2006; Malnoy et al. 2007; Meur et al. 2008; Zhang et al. 2010; Silva et al. 2015). Importantly, expression of *AtNPR1* in most crop species did not result in strong collateral effects on plant growth and development, which is crucial for utilizing this gene in genetic engineering of broad-spectrum disease resistance in crops.

We have previously generated a group of transgenic citrus lines expressing the *AtNPR1* gene (Zhang et al. 2010). Characterization of these transgenic lines revealed that overexpression of *AtNPR1* in citrus increases resistance to citrus canker. Here, we further tested these transgenic lines for HLB responses and identified three lines that exhibit vegetatively inheritable tolerance to HLB. We also analyzed AtNPR1 levels in the HLB-tolerant transgenic lines. Our results indicate that expression of high levels of *AtNPR1* in citrus is able to provide tolerance to HLB under strong disease pressure in the greenhouse.

Materials and Methods

Plant materials and growth conditions

Nine transgenic 'Duncan' grapefruit lines and seven transgenic 'Hamlin' sweet orange lines expressing *AtNPR1* have been previously reported (Zhang et al. 2010). The transgenic plants were maintained in standard temperature-controlled greenhouses at the Citrus Research and Education Center of the University of Florida.

HLB inoculation

HLB inoculation was conducted by simply moving plants into a *C*Las-infected psyllid room. The plants were inoculated in a room with thousands of *C*Las-infected psyllids for up to two-month intervals. Plants with new feather flush that would attract psyllids for egg deposition, resulting in later nymph emergence, were put into the psyllid room. Development of nymphs on each plant was recorded. Afterwards, the psyllids were removed and the plants were moved to an immediate screenhouse for about two weeks to ensure psyllid removal. The plants were then maintained in the greenhouse for symptom development. Plants that developed severe HLB symptoms were destroyed (Folimonova et al. 2009; Lee et al. 2015; Ammar et al. 2016).

Real-time quantitative PCR analysis

Approximately 0.3 g citrus leaf petioles and/or midribs in 3 mL extraction buffer (50 mM Tris-HCl, pH 9.0, 0.1 M NaCl, 10 mM EDTA, 10 mM freshly prepared DTT) were ground with a Kleco Tissue Pulverizer (Garcia Machine, Visalia, CA) for 30 sec. The extract (1,300 μL) was transferred into a 2 mL microfuge tube. After adding 90 μL 20% SD, the mixture was incubated for 60 min at 65°C, followed by addition of 500 μL of 5 M potassium acetate and incubation for 20 min on ice. After centrifugation at 16,000 × *g* for 10 min, 500 μL supernatant was transferred to a new tube, to which 500 μL cold isopropanol was added. After mixing, DNA was precipitated by centrifugation at 16,000 × *g* for 10 min. The pellet was washed with 70% ethanol, dried, and dissolved in 100 μL distilled water. Real-time quantitative PCR (qPCR) was performed as previously described (Li et al. 2006). Reactions were run and analyzed with the Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions. The primers used were described in Li et al. (2006).

Immunoblot analysis

Protein was extracted from fully expanded citrus leaves using a Plant Total Protein Extraction Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol. Briefly, ~0.1 g leaf tissues were ground in liquid nitrogen and suspended with 1.5 mL of pre-chilled (-20°C) methanol supplemented with protease inhibitors. The mixture was incubated for 5 min at -20°C with periodic vortexing. After centrifugation at 16,000 × *g* for 5 min at 4°C, the supernatant was carefully discarded. The pellet was extracted two additional times with the methanol solution and then suspended in 1.5 mL pre-chilled (-20°C) acetone. The mixture was incubated for 5 min at -20°C. After centrifugation at 16,000 × *g* for 5 min at 4°C, the supernatant was carefully discarded. The pellet was allowed to air dry for 10 min at room temperature and the mass of the pellet was determined. After adding protein extraction reagent (4 μL/mg pellet), the pellet was

completely broken up by vortexing and the mixture was incubated for 15 min at ambient temperature with intermittent vortexing. After centrifugation at 16,000 × *g* for 30 min, the supernatant was mixed with 2 × sample buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol, 200 mM DTT, and 0.4% bromophenol blue). The protein samples were heated at 60°C for 10 min, loaded onto an 8% SDS-PAGE gel, and transferred to nitrocellulose after electrophoresis. The blot was probed with an anti-NPR1 antibody (Ding et al. 2016) and a horse-radish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Dallas, TX) followed by chemiluminescence with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

Conversion from juvenile to mature tissues

The coding region of the *Citrus clementine FLOWERING LOCUS T3* (*CcFT3*) gene (kindly provided by Drs. Gloria Moore and Ed Stover, University of Florida) was cloned into a *Citrus tristeza virus* (CTV)-based vector using the PacI and StuI restriction sites. The resulting construct was introduced into the *Agrobacterium tumefaciens* strain COR308 by heat shock transformation at 42°C for 5 min. Agro-inoculation of *Nicotiana benthamiana* was conducted as previously described (Ambrós et al. 2011). Briefly, Agrobacteria were first washed with a wash buffer (10 mM MES-NaOH, 10 mM $MgCl_2$, pH 5.6) and then resuspended in an induction buffer (10 mM MES-NaOH, 10 mM $MgCl_2$, pH 5.6 with 150 μM acetosyringone) to an OD_{600} of 0.8 to 1. After 3 to 5 hr of induction in the buffer, the Agrobacterium suspension was infiltrated into fully expanded *N. benthamiana* leaves using a needleless 1 mL syringe. About 1.5 to 2 months later, CTV-CcFT3 recombinant virions were purified from systemically infected *N. benthamiana* leaves and bark flap inoculated into one-year-old *C. macrophylla* seedlings (Robertson et al. 2005), which began blooming in about five months. The transgenic plants were then inoculated by grafting buds from the *C. macrophylla* trees that have bloomed.

Results

Identification of citrus AtNPR1 transgenic lines tolerant to HLB

We have previously shown that transgenic citrus plants overexpressing the *AtNPR1* gene exhibited increased resistance to citrus canker (Zhang et al. 2010). To test if overexpression of *AtNPR1* could also provide resistance or tolerance to HLB, we evaluated HLB responses of the transgenic lines (nine in the 'Duncan' grapefruit background and seven in the 'Hamlin' sweet orange background) by psyllid inoculation of HLB under high levels of inoculum pressure. Plants were subjected to large numbers of *C*Las-infected psyllids for long periods of time and flush tissues were subjected to numerous infected adults that resulted in egg production and nymph development. Non-transgenic control and non-tolerant transgenic 'Duncan' grapefruit and 'Hamlin' sweet orange plants developed strong leaf symptoms and reduced growth with some shoot growth becoming terminal. However, after more than six rounds of HLB inoculation, three independent transgenic lines appeared to be HLB tolerant based on greatly reduced leaf symptoms and continued normal growth. To ensure that this level of tolerance would continue as the plants continued to be propagated by buds, the putative HLB-tolerant lines were budded to 'Volkamer' and 'Carrizo' rootstocks (Fig. 1). Progeny plants (2nd generation) were produced from the original transgenic plants (1st generation) by grafting, and 3rd generation progenies were produced from the 2nd generation plants. All progeny plants were inoculated in the infected psyllid room only after producing new flushes. Nymph-infested plants were labeled and returned back to the greenhouse for symptom development and qPCR analysis of *C*Las titers. qPCR-negative plants were inoculated again in the infected psyllid room until all plants became *C*Las positive. The three independent transgenic lines, 'Duncan' 57-28, 'Hamlin' 13-3, and 'Hamlin' 13-29, have continued growing normally after numerous rounds of inoculation over a period of nine years (2008 to 2017) and only occasionally exhibited mild HLB symptoms (Fig. 2a, b). All progenies of these transgenic lines exhibited high levels of tolerance to HLB (Fig. 2b). These results indicate that the three transgenic lines are truly tolerant to HLB.

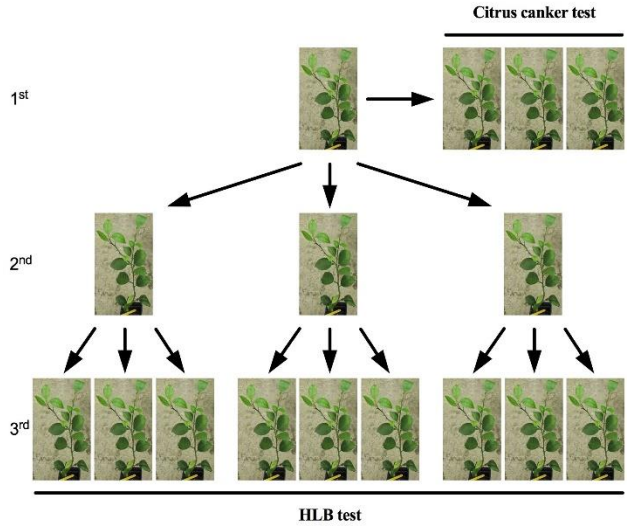

Fig. 1. Schematic diagram of the HLB test procedure in this study. The original transgenic plants (1st generation) were propagated and the progenies were used for the citrus canker test (Zhang et al. 2010). The original transgenic plants were directly screened for HLB resistance or tolerance based on disease symptoms. Transgenic plants showing no or mild HLB symptoms were propagated. The progenies (2nd generation) were tested. Transgenic lines that appeared to be tolerant in the 1st generation, but when propagated some 2nd generation plants were not tolerant, were discarded. Those lines with all 2nd generation plants showing no or mild symptoms were further propagated and the 3rd generation progenies were similarly tested. Transgenic lines with all the 3rd generation progenies retaining the same level of tolerance were considered tolerant.

(a)

(b)

	'Duncan' 57-28	'Hamlin' 13-3	'Hamlin' 13-29
1st generation	0.5	0.5	0.5
2nd generation	Two 0, one 0.5	One 0.5	Six 0, one 1
3rd generation	Twelve 0, one 1	Two 0	Twelve 0, one 0.5, three 1, one 2

Fig. 2. HLB disease scores of the three HLB-tolerant transgenic lines. (a) Phenotypes of HLB disease symptoms to which different disease scores were assigned after inoculation with HLB using *C*Las-infected psyllids. Score = no symptoms, Score 1 = very mild symptoms on some leaves, Score 2 = clear symptoms on most leaves, and Score 3 = severe symptoms on most leaves. Scores 0.5, 1.5, and 2.5 were also used when intermediate phenotypes were observed. (b) Three independent transgenic lines, one in the 'Duncan' background and two in the 'Hamlin' background, exhibited robust tolerance to HLB.

CLas titers in the transgenic lines tolerant to HLB

The majority of the 2nd and 3rd generation progeny plants of the three HLB-tolerant lines did not show any HLB symptoms (Fig. 2b). To confirm these plants were HLB positive, we determined the *C*Las titers in the plants by qPCR with primers specific for *C*Las 16S rDNA and verified the quality of DNA extraction using primers specific for the positive internal control, the citrus cytochrome oxidase (*COX*) gene (Li et al. 2006). Using these primers, we usually obtain a threshold cycle (Ct) value of about 21 to 24 for *C*Las and a Ct value of about 15 to 16 for *COX* from plants with severe HLB symptoms. For the three HLB-tolerant lines, the *C*Las Ct values varied for leaf tissues collected at different times, even though the *COX* Ct values were consistently about 13 to 15. Nevertheless, the *C*Las Ct values for the three HLB-tolerant lines were generally less than 30 (Fig. 3), indicating that these plants have been infected by *C*Las. The HLB-tolerant line 'Hamlin' 13-29 is shown in Fig. 3. The original plant of the transgenic line (Fig. 3a), which was produced in 2008, has been *C*Las positive for seven years and has continued growing normally. Six progeny plants (Fig. 3b to g) are *C*Las positive (Ct < 21-30) and do not have any HLB symptoms.

NPR1 protein levels in the transgenic lines tolerant to HLB

We used immunoblotting with a specific anti-AtNPR1 antibody (Ding et al. 2016) to analyze AtNPR1 levels in the transgenic lines. As shown in Fig. 4, all three HLB-tolerant transgenic lines accumulated significant amounts of AtNPR1. Interestingly, although the three lines exhibited comparable levels of HLB tolerance, AtNPR1 level in 'Duncan' 57-28 was much lower than those in 'Hamlin' 13-3 and 'Hamlin' 13-29 (Fig. 4), suggesting that the threshold levels of AtNPR1 required for conferring robust HLB tolerance may be different for the two genotypes.

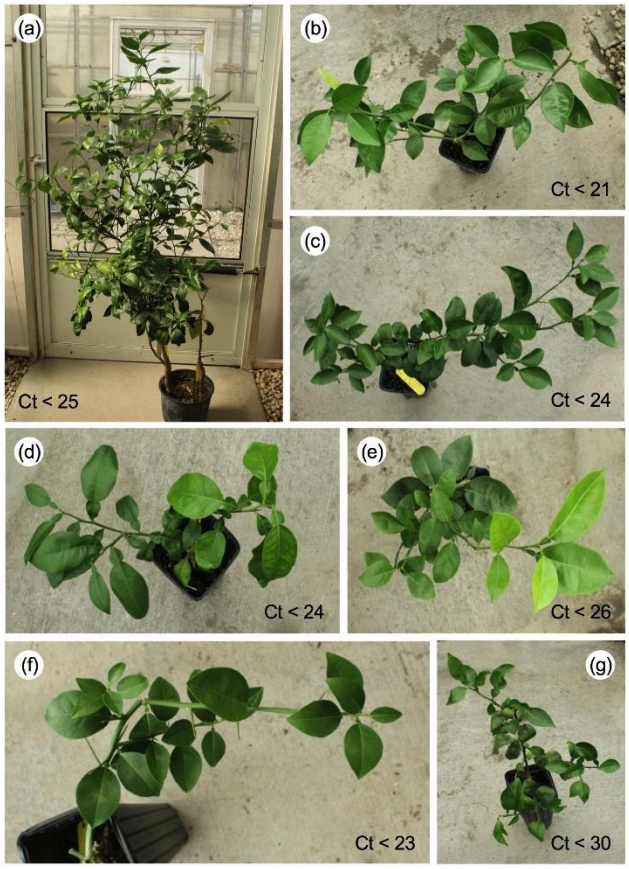

Fig. 3. A representative HLB-tolerant transgenic line. (a) The original plant of the transgenic line 'Hamlin' 13-29, which was produced in 2008, has been *C*Las positive (indicated by the Ct value) for seven years and has continued growing normally. (b) to (g) Six progeny plants of the transgenic line. These plants are also *C*Las positive (indicated by the Ct values) but do not have HLB symptoms.

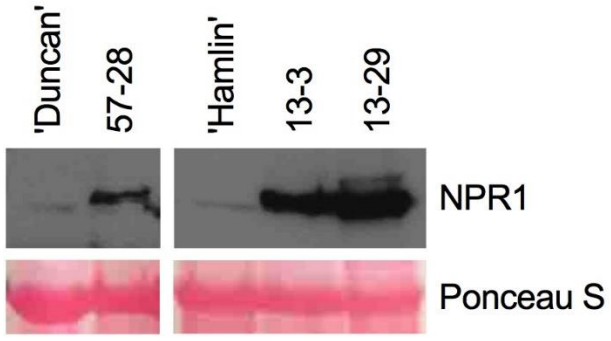

Fig. 4. AtNPR1 protein levels in the three HLB-tolerant transgenic lines. The AtNPR1 protein levels in the three HLB-tolerant lines were detected with a specific anti-AtNPR1 antibody. The Ponceau S staining of Rubisco was used as the loading control.

CcFT3-mediated promotion of flowering of the HLB-tolerant transgenic lines

The transgenic plants were originally generated from juvenile (epicotyl) explants (Zhang et al. 2010). It was unclear if the transgenic plants could develop flowers normally. To test this, we attempted to promote transition from vegetative to reproductive phase in the transgenic plants by expression of the *CcFT3* gene, which encodes an ortholog of the *A. thaliana* FT protein. FT and its orthologs in other plant species including citrus are the central component of the flowering signal cascade (Corbesier et al. 2007; Lin et al. 2007; Nishikawa et al. 2007, Tamaki et al. 2007; Kong et al. 2010; Hecht et al. 2011; Imamura et al. 2011; Muñoz-Fambuena et al. 2011; Lv et al. 2012). Ectopic expression of the *C. unshiu FT* (*CuFT*) gene driven by the *Cauliflower mosaic virus* 35S promoter has been shown to promote flowering in trifoliate orange (Endo et al. 2005). However, constitutive overexpression of the *CuFT* gene resulted in a dwarfed aberrant morphology (Endo et al. 2005), indicating detrimental effects on plant growth and development. Recently, it has been reported that expression of the *Citrus sinensis FT* gene in juvenile *C. excelsa* seedlings using a *Citrus leaf blotch virus*-based vector stimulated flowering without alteration of the plant architecture, leaf, flower, and fruit morphology (Velazquez et al. 2016). In our study, we inoculated the three HLB-tolerant transgenic lines with a CTV-based construct carrying the *CcFT3* gene. All inoculated plants have developed flowers and representative plants of the three transgenic lines are shown in Fig. 5. This result indicates that the *AtNPR1* gene does not affect flower development when overexpressed in citrus plants.

Fig. 5. Representative flowering plants. Representative flowering plants of the three HLB-tolerant transgenic lines 'Duncan' 57-28, 'Hamlin' 13-3, and 'Hamlin' 13-29. Rep: replicate. The photos in the bottom row are enlarged flowers or fruits.

Discussion

In this study, we screened the previously generated transgenic 'Duncan' grapefruit and 'Hamlin' sweet orange expressing the *AtNPR1* gene and identified three independent lines (one line in 'Duncan' grapefruit and two lines in 'Hamlin' sweet orange) that are highly tolerant to HLB. The transgenic plants were produced in 2008 and have since been under evaluation for HLB resistance and tolerance. The majority of these plants were eliminated over the years due to severe HLB symptoms. However, three of the transgenic lines were positive for *C*Las, but had occasional and minor leaf symptoms and have continued growing normally (Fig. 2, 3). Importantly, all progenies vegetatively propagated from the three original transgenic plants retained the same level of HLB tolerance (Fig. 2, 3). The three transgenic lines also accumulate high levels of AtNPR1 (Fig. 4), and have previously been shown to be resistant to citrus canker (Zhang et al. 2010). These results together convincingly demonstrate that overexpression of *AtNPR1* is able to provide tolerance to HLB.

In a previous report, Dutt et al. (2015) showed that some transgenic 'Hamlin' sweet orange lines highly expressing the *AtNPR1* gene remained *C*Las free for 24 and 36 months in greenhouse and field tests, respectively, thus concluded that *AtNPR1*, when overexpressed in citrus, is able to provide resistance to HLB. Under extremely high levels of infection pressure by many *C*Las-infected psyllids, our transgenic plants became infected. However, three lines exhibited only minor symptoms and continued to grow similarly to uninfected plants. Thus, we argue that multiple rounds of HLB inoculation of several sets of progenies vegetatively propagated from the original transgenic plants are necessary for identifying true HLB resistance. In our experiments, some transgenic lines remained *C*Las free even after six rounds of HLB inoculation, but ultimately became *C*Las positive under strong pathogen pressure with the optimized inoculation method. It is possible that the HLB-resistant transgenic lines identified by Dutt et al. (2015) escaped inoculation under the level of disease pressure in the field. In any case, the findings made by Dutt et al. (2015) are consistent with the results from our long-term greenhouse evaluation.

Our results are also in agreement with the findings reported by Wang, Zhou, et al. (2016), who evaluated transcriptome differences between two closely related cultivars after HLB infection: HLB-tolerant 'Jackson' grapefruit-like-hybrid trees and HLB-susceptible 'Marsh' grapefruit trees. This unique comparison allowed the authors to identify genes differentially expressed between the HLB-tolerant and HLB-susceptible citrus genotypes. It was reported that four *AtNPR1*-like genes were expressed to significantly higher levels in the HLB-tolerant genotype than in the HLB-susceptible genotype. The *AtNPR1*-like gene-mediated defense signaling may contribute to the HLB-tolerant phenotype of 'Jackson' (Wang, Zhou et al. 2016). Our results are further supported by the effectiveness of the SAR signaling molecule salicylic acid (SA) and its analogs in suppressing HLB disease progression in the field (Li et al. 2016; Li et al. 2017). In Arabidopsis, SA not only induces the *AtNPR1* gene expression but also enhances the activity of AtNPR1 through diverse mechanisms (Pajerowska-Mukhtar et al. 2013). Conversely, AtNPR1

transduces the majority of the SA-activated defense signaling. Thus, the citrus NPR1 may directly mediate the suppressive effects of SA and its analogs on HLB disease progression in citrus, and as previously reported in Arabidopsis (Cao et al. 1998; Friedrich et al. 2001), the HLB tolerance of the AtNPR1-overaccumulating citrus transgenic plants may be associated with faster and stronger responses to *C*Las infection and SA. Further investigations are needed to fully understand the mechanisms mediating the HLB tolerance.

Although the HLB-tolerant transgenic lines identified in this study could potentially be used in the citrus industry, they are genetically modified organisms (GMOs) that will not only have to go through the regulatory approval process, but will also have to face the negative attitudes of some consumers towards GMO products (Magnusson and Hursti 2002). Nevertheless, the success in generating HLB tolerance in citrus using the *AtNPR1* gene identified a genetic target for recreating the phenotype using technologies such that the resulting trees will no longer be considered GMOs. For instance, cisgenic/intragenic approaches may be utilized to increase the expression of the citrus *NPR1* that has been shown to be functional (Chen et al. 2013; Espinoza et al. 2013). An efficient citrus intragenic vector is already available for this purpose (An et al. 2013). Alternatively, the revolutionary Clustered Regularly Interspaced Short Palindromic Repeats/associated nuclease Cas9 (CRISPR/Cas9) gene-editing technology may be employed to inactivate negative regulators of the citrus *NPR1*, or to delete negative elements in the promoter of the citrus *NPR1* gene (Wang, Russa, et al. 2016). In this regard, a highly efficient citrus transgene-free CRISPR/Cas9 technique needs to be developed.

Acknowledgments

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