Reduced Susceptibility to *Xanthomonas citri* in Transgenic Citrus Expressing the FLS2 Receptor From *Nicotiana benthamiana*

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Submitted 17 September 2015. Accepted 6 November 2015.

Overexpression of plant pattern-recognition receptors by genetic engineering provides a novel approach to enhance plant immunity and broad-spectrum disease resistance. Citrus canker disease associated with *Xanthomonas citri* is one of the most important diseases damaging citrus production worldwide. In this study, we cloned the *FLS2* gene from *Nicotiana benthamiana* cDNA and inserted it into the binary vector pBinPlus/ARS to transform Hamlin sweet orange and Carrizo citrange. Transgene presence was confirmed by polymerase chain reaction (PCR) and gene expression of *NbFLS2* was compared by reverse transcription quantitative PCR. Reactive oxygen species (ROS) production in response to flg22_{Xcc} was detected in transgenic Hamlin but not in nontransformed controls. Low or no ROS production was detected from nontransformed Hamlin seedlings challenged with flg22_{Xcc}. Transgenic plants highly expressing *NbFLS2* were selected and were evaluated for resistance to canker incited by *X. citri* 3213. Our results showed that the integration and expression of the *NbFLS2* gene in citrus can increase canker resistance and defense-associated gene expression when challenged with *X. citri*. These results suggest that canker-susceptible *Citrus* genotypes lack strong basal defense induced by *X. citri* flagellin and the resistance of these genotypes can be enhanced by transgenic expression of the flagellin receptor from a resistant species.

Plants use pattern-recognition receptors to detect potential pathogens by recognizing pathogen-associated molecular patterns (PAMPs) and eliciting PAMP-triggered immunity (PTI) against pathogen growth and disease development. PTI is associated with PAMP recognition and activation of plant plasma membrane receptors (Gómez-Gómez et al. 2001; Jones and Dangl 2006). PAMPs such as bacterial flagellin are largely conserved across a wide range of bacteria, as these molecules are essential for motility and movement. Perception of bacterial flagellin by AtFLS2 (flagellin sensing 2) in *Arabidopsis thaliana* is well studied and the conserved peptide flg22 induces the expression of many defense-related genes (Zipfel et al. 2004). FLS2 interacts with the receptor kinase BAK1 by forming a functional FLS2/BAK1 complex, which is subsequently followed by calcium ions (Ca²⁺) influx, reactive oxygen species (ROS) production, and

mitogen-activated protein kinase (MAPK) pathway activation (Gómez-Gómez et al. 2001; Jones and Dangl 2006).

Mutagenesis and crystal structure studies on AtFLS2 *A. thaliana* revealed that some amino acids, including 366T, 392H, and 414D in the extracellular leucine-rich repeat (LRR) domain and 1064G in the kinase domain, are essential for binding to flg22 (Dunning et al. 2007; Gómez-Gómez et al. 2001; Sun et al. 2013). FLS2 from different plant species have specificities that permit recognition of flagellin peptides from different bacterial species (Chinchilla et al. 2006; Robatzek et al. 2007). SIFLS2 from tomato (*Solanum lycopersicum*) and NbFLS2 from *N. benthamiana* can perceive the 22 and 15 amino-acid peptides derived from *Pseudomonas syringae* flagellin; however, SIFLS2 but not NbFLS2 can recognize the 15 amino-acid peptide derived from *Escherichia coli* flagellin (Robatzek et al. 2007). AtFLS2 and SIFLS2 have been shown to exhibit species-specific differences in the recognition of shortened or modified flg22 ligands through domain-swapping studies (Mueller et al. 2012). OsFLS2 from rice (*Oryza sativa*) was discovered to be responsive only to a specific flagellin from the incompatible strain *P. avenae* (Che et al. 2000; Felix et al. 1999). VvFLS2 from grapevine (*Vitis vinifera*) has been shown to differentially recognize flg22 epitopes from a growth-promoting bacterium and plant-pathogenic bacteria (Takai et al. 2008). Though FLS2 orthologs from different plants have specificities in recognition of specific bacterial flagellins, some bacterial flagellins are widely perceived by FLS2 orthologs characterized from different plant species, including rice (OsFLS2), *N. benthamiana* (NbFLS2), tomato (SIFLS2), and grapevine (VvFLS2) (Chakravarthy et al. 2010; Robatzek et al. 2007; Takai et al. 2008; Trdá et al. 2014). It has been reported that VvFLS2 from grapevine can complement *A. thaliana* fls2 mutant function and AtFLS2 from *A. thaliana* is functional in transgenic tobacco (Trdá et al. 2014; Robatzek et al. 2007).

Citrus canker associated with *X. citri* is one of the most important diseases affecting citrus production worldwide, leading to yield losses, poor fruit quality, and trade barriers. The disease affects leaves, stems, and fruits, and symptoms consist of roughly circular water-soaked lesions that cause defoliation, dieback, and fruit drop (Brunings and Gabriel 2003). Many commercial citrus varieties, including sweet orange [*Citrus sinensis* (L.) Osbeck], grapefruit (*C. paradisi* Macf), and lemon [*C. limon* (L.) Burm. f.], are susceptible to canker disease (Gottwald et al. 1993). Recently, the susceptible citrus genotypes ‘Duncan’ grapefruit and ‘Navel’ sweet orange have been shown to have no significant ROS production or PTI/effector-triggered immunity-associated gene induction following flg22_{Xcc} treatment (Shi et al. 2014). In contrast, resistant genotypes such as ‘Nagami’ kumquat and ‘Sun Chu Sha’ mandarin show higher ROS production following flg22_{Xcc} treatment (Shi et al. 2014).

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*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures are published online.

Genetic engineering is a promising strategy to complement classical citrus breeding and enhance disease resistance for susceptible commercial varieties. Several resistance (*R*) genes have been transgenically introduced into plants for disease control. The *Bs2* pepper gene conferred resistance to bacterial spot disease when expressed in tomato (Tai et al. 1999). Transgenic tobacco expressing the tomato gene *Pto* showed enhanced resistance to disease incited by *Pseudomonas syringae* pv. *tabaci* (Thilmony et al. 1995). However, *R* gene-induced resistance provides resistance only to particular pathogen strains and rarely confers broad-spectrum disease resistance (McDonald and Linde 2002). In addition, it is easily overcome by pathogens via mutation or gene transfer to acquire new effectors that counteract plant resistance (Leach et al. 2001).

Overexpression of PAMP receptors has been proposed as a way to enhance disease resistance in transgenic plants. Strong immune response was induced by flagellin and flg22 in transgenic rice through overexpression of OsFLS2; however, no increased disease resistance was observed when infiltrated with bacteria (Takai et al. 2008). The bacterial elongation factor Tu (EF-Tu) is recognized by the EF-Tu receptors (EFR) from *Brassicaceae* and *Solanaceae* species and some rice cultivars (Boller and Felix 2009). It has been demonstrated that transgenic expression of *EFr* from *A. thaliana* conferred responsiveness to bacterial EF-Tu in *N. benthamiana* and tomato, enhancing disease resistance to a range of phytopathogenic bacteria from different genera (Lacombe et al. 2010). In addition, expression of rice *XA21* in sweet orange, tomato, and banana enhanced resistance to *X. citri*, *Ralstonia solanacearum*, and *X. campestris* pv. *musacearum*, respectively (Afroz et al. 2011; Mendes et al. 2010; Tripathi et al. 2014). Heterologous expression of these PAMPs has been proven to enhance broad-spectrum disease resistance through strengthening the plants' own innate immune system. We have previously reported that *Agrobacterium*-mediated transient expression of flagellin from 'Candidatus Liberibacter asiaticus' and 'Ca. L. solanacearum' in *N. benthamiana* is sufficient to induce ROS production and PTI-associated gene expression (Hao et al. 2014; Zou et al. 2012). Compared with flg22_{Las} and flg22_{Lso}, flg22_{Xcc} can induce PAMP-associated gene expression at a

much lower concentration and elicit a stronger cell-death response following transient expression in *N. benthamiana* (Chinchilla et al. 2006; Zou et al. 2012). Hamlin sweet orange (*C. sinensis* (L.) Osbeck) is one of the most important citrus scion cultivars in Florida and is very susceptible to canker disease. Carrizo citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L.) is an important rootstock and is also susceptible to canker. In the present study, we transgenically expressed *NbFLS2* in Hamlin and Carrizo. We determined that ROS production was significantly induced in transgenic citrus exposed to flg22_{Xcc}. We showed that transgenic citrus plants with high expression levels of *NbFLS2* displayed enhanced disease resistance to citrus canker via improving plant basal defense through interaction of bacterial flagellin and *NbFLS2*. We demonstrated that the PTI marker gene *WRKY22* and defense-related gene *PR1* were also activated in transgenic citrus plants carrying highly expressed *NbFLS2* after exposure to *X. citri*.

RESULTS

Hamlin and Carrizo citrus plants transformed with *NbFLS2*.

A full-length *NbFLS2* coding sequence was amplified from cDNA and was inserted into pBinPlus/ARS transcribed from a double 35S promoter. A total of 17 independent kanamycin-resistant Hamlin plants were obtained by *Agrobacterium*-mediated transformation. Polymerase chain reaction (PCR) primers *NbFLS2*-For and *Nos*-Rev (Table 1), located in *NbFLS2* and the *Nos* terminator of pBinPlus/ARS, were used for PCR amplification. Ten of these plants were shown to contain the *NbFLS2* transgene (Supplementary Fig. S1A). Similarly, over 30 transgenic Carrizo plants were confirmed to contain the *NbFLS2* gene. The PCR-positive *NbFLS2* plants were further evaluated.

Difference in transgene expression among the transgenic citrus plants.

RNA from the *NbFLS2*-positive transgenic plants was isolated and amplified by conventional reverse transcription PCR (RT-PCR), confirming transgene transcription in these plants (data not shown). The relative levels of transcription in these

Table 1. Primers used in this study

Gene name	Primer sequence	Amplicon size (bp)
For transgenic plants		
<i>NbFLS2</i> -For	TCAAATGGTGGATGACTGGA	930
<i>Nos</i> -Rev	TTTGCGCGCTATATTTGT	
For <i>NbFLS2</i> cloning		
<i>NbFLS2</i> -ORF-For	TCTCCCGGGAACCAACAATGTCACAGACAG	3,500
<i>NbFLS2</i> -ORF-Rev	TCGACTAGTTCAAATTGCATCGTTTACCAACAAG	
For gene expression by real time polymerase chain reaction		
<i>NbFLS2</i> -RT-5'	GGAGAAAGGGAACCTTGGACAATA	97
<i>NbFLS2</i> -RT-3'	ACCACTAGCAACCGAACTAAA	110
<i>CsFLS2</i> -RT-5'	CAGGACTGGATGAAGAGAATGG	
<i>CsFLS2</i> -RT-3'	CAGCCTAGGGTCTGTGATTG	103
<i>CsFLS2</i> -L-RT-5'	CAACCAACATCAGTCCACAATAAG	
<i>CsFLS2</i> -L-RT-3'	CAAAGAGGGTGACTACAGCTATC	100
<i>CsPR1</i> -RT-5'	ACAAACACACATCTCCGAAATGA	
<i>CsPR1</i> -RT-3'	TTGAAATGAGCAGCAGCAAAA	106
<i>CsGST1</i> -RT-5'	TCAAGGGCAATCACCCAATAC	
<i>CsGST1</i> -RT-3'	CCACCTCCTTCCATACCAATAAC	97
<i>CsWRKY22</i> -RT-5'	GGCCTAAGCTTTCCGATTCT	
<i>CsWRKY22</i> -RT-3'	GTCACCCTTTGCACTACTCTT	80
<i>CsGAPC2</i> -RT-5'	TCTTGCTGCTTTGAATGGA	
<i>CsGAPC2</i> -RT-3'	TGTGAGGTCAACCACTGCGACAT	
For bacterial growth by pthA detection		
VM3	GCATTTGATGACGCCATGAC	151
VM4	TCCCTGATGCCTGGAGGATA	

plants were compared by RT-quantitative (q)PCR, and highly variable expression was observed. In particular, transgenic Hamlin plants H5, H6, and H16 displayed high transcription levels, while transgenic plants H2, H10, H13, and H17 showed low transcription levels (Fig. 1A). The absence of *NbFLS2* mRNA in transgenic plant H12 may be due to transgene insertion position or gene silencing. Similarly, conventional RT-PCR and real time qPCR were performed to compare gene expression in transgenic Carrizo plants. Transgenic Carrizo plants C11, C51, C57, and C66 showed high *NbFLS2* expression level while C35 and C38 have relatively low expression levels (Fig. 1B).

Characteristics of citrus FLS2 orthologs and their expression in nontransformed and *NbFLS2* transgenic citrus plants.

To identify *FLS2* orthologs in citrus, a blast search was performed against the *NbFLS2* protein (Joint Genome Institute [JGI] Phytozome database and the National Center for Biotechnology Information [NCBI]). Two citrus *FLS2* orthologs were identified from Valencia sweet orange using the NCBI database, which showed high homology (99%) with *FLS2* homologs from *C. clementina* (JGI Phytozome database), and one of them was designated as *CsFLS2*. Gene *CsFLS2* encodes a protein with 1,194 amino acids sharing 54% identity with *A. thaliana* *AtFLS2* and 61% identity with *N. benthamiana* *NbFLS2* (Supplementary Fig. S2). A blast search of *C. sinensis* sequences from the JGI Phytozome database using *NbFLS2* showed several candidates that shared significantly lower identity (35%) with *AtFLS2* from *Arabidopsis* and *NbFLS2* from *N. benthamiana*. One of them (orange1.1 g000859m) encodes 1,251 amino acids and was designated as *CsFLS2*-like (*CsFLS2*-L) for further analysis. Sequence alignment analysis showed *CsFLS2* shares the typical *FLS2* LRR-RK structure. The LRR domain of *CsFLS2* consists of 28 LRR tandem repeats, similar to *AtFLS2* and *LeFLS2*. In contrast, the LRR domain of *CsFLS2*-L consists of 31 LRR tandem repeats with

highly variable sequences. It is noteworthy that *CsFLS2* shares all the critical amino acids for *FLS2* function, while *CsFLS2*-L contains only some of them. Phylogenetic analysis showed that the *CsFLS2* protein is in the same cluster with *FLS2* homologs from poplar and *V. vinifera* with a high level of confidence. *CsFLS2*-L was shown to be somewhat more distantly associated with the other genes in the phylogenetic tree (Fig. 2). These data suggest that *CsFLS2* is a promising *FLS2* ortholog and *CsFLS2*-L may or may not be a *FLS2* ortholog.

To investigate citrus *FLS2* ortholog expression in transgenic plants expressing *NbFLS2* and control plants, primers were designed in the unique regions of *NbFLS2*, *CsFLS2*, and *CsFLS2*-L (Table 1). RT-qPCR indicated low levels of expression for *CsFLS2* and *CsFLS2*-L compared with *NbFLS2* in Hamlin and Carrizo transgenic plants with native gene expression similar to nontransgenic control plants (Supplementary Fig. S3). The transgenic plants showed normal leaf morphology and overall appearance.

High ROS production induced in some of the transgenic citrus.

To test whether nontransgenic Hamlin can produce ROS response to flg22_{Xcc}, leaves from over 40 2-month-old Hamlin seedlings were tested and no marked ROS production was detected in most of them, with relatively low ROS production observed from two seedlings (Fig. 3A). In contrast, transgenic Hamlin plants displayed ROS production in response to flg22_{Xcc} that ranged up to 14-fold that of the nontransformed Hamlin control. Transgenic Hamlin plant *FLS2*-H5 showed the highest levels of ROS production, and transgenic plants *FLS2*-H6 and H10 showed intermediate levels (Figs. 3C and 4A), and ROS production correlated with the *NbFLS2* expression levels in these transgenic plants. These results suggest that *FLS2* receptors from sweet orange poorly recognize the *X. citri* flagellin peptide, even though *CsFLS2* and *CsFLS2*-L RNA were detected in transgenic and nontransgenic plants. In part, this may result from *CsFLS2*-L showing low homology with known

Fig. 1. Gene expression in citrus. **A**, Transgenic Hamlin expression of *NbFLS2* and **B**, transgenic Carrizo expression of *NbFLS2*. Expression fold change is relative to the lowest *NbFLS2* expression tested, which was used as a calibrator due to its low abundance.

functional FLS2 receptors and lacking critical amino acids essential for FLS2 function.

A total of 20 nontransgenic Carrizo seedlings were also tested for ROS response. A somewhat higher peak ROS production was produced from a few Carrizo seedlings compared with the Hamlin seedlings; however, no marked ROS was detected in most Carrizo seedlings and transgenic Carrizo had much higher ROS production (Fig. 3). Compared with transgenic Hamlin, higher ROS production was detected from transgenic Carrizo plants (Fig. 3D), which may reflect a contribution from greater Carrizo basal response, also reflected by somewhat higher ROS production in nontransgenic seedlings (Fig. 3B). One ROS peak was detected in most of the Carrizo transgenic plants. However, two ROS peaks were repeatedly detected from transformed Carrizo plant C57 (Fig. 4B). A typical ROS peak was initiated about 5 min after treatment with flg22_{Xcc} and returned to the basal level after 35 mins. However, in transgenic plant C57, before ROS production returned to the basal level, a second ROS peak began 28 min after treatment with flg22_{Xcc} (Fig. 4B). Overall, these data suggest that FLS2 orthologs in sweet orange and Carrizo interact weakly with *X. citri* flagellin. In contrast, transgenic expression of *NbFLS2* activated a strong ROS response involving the citrus PTI pathway. This weak to nonexistent response from native FLS2 orthologs may result in high susceptibility of Hamlin and Carrizo to citrus canker caused by *X. citri*.

Reduced susceptibility to *X. citri* in the transgenic citrus with high ROS.

To determine whether the *NbFLS2* transgene influences the susceptibility of sweet orange cultivars to *X. citri*, eight independent transgenic Hamlin plants and 10 independent transgenic Carrizo plants were inoculated with the pathogen. Several nontransformed plants and *FLS2* PCR-negative plants of each cultivar were used as controls. The effectiveness of the transgene in reducing disease development was not significant with infiltration at the concentration of 10⁷ or 10⁶ CFU per milliliter. Slightly reduced canker symptoms were shown in transgenic citrus with lower titer infiltration (10⁴ or 10³ CFU per milliliter) (Supplementary Fig. S4). *X. citri* growth was also assessed at various time points after leaf infiltration at 10⁶ CFU per milliliter in transgenic plants and nontransgenic control. No obvious *X. citri* growth difference was observed between transgenic and nontransgenic leaf disc samples collected at 0, 3, 6, 9, and 12 day after inoculation (dai) (data not shown). Therefore, leaf disc samples were taken at 2 h, 1, 2, 3, 5, and 7 dai in a second experiment. Bacterial population decreased at 1 dai in Hamlin transgenic plants H5 and H6 (Supplementary Fig. S5), but growth resumed and was not obviously different from controls. However, with spray inoculation at 10⁶ CFU per milliliter, transgenic plants with high ROS production and high *NbFLS2* expression showed marked disease resistance 4 weeks after inoculation (Fig. 5A and D). Furthermore, canker lesion development was greatly reduced in Hamlin transgenic plants H5 and H6 and Carrizo transgenic plant C11 compared with other transgenic plants and control plants (Fig. 5E and F). With spray inoculation, all tested transgenic Hamlin and Carrizo expressing *NbFLS2* showed reduced canker development as compared with control plants. Based on statistical analysis by one-way analysis of variance and Tukey's-B posttest, the canker lesion development level is significantly less in transgenic plant expressing *NbFLS2* than in control plants (Fig. 6).

To further investigate whether PTI or defense-associated gene expression was affected after *X. citri* challenge, *WRKY22*, and *GST1* expression, which were induced in *Arabidopsis* and citrus with flg22 treatment, were investigated as markers of PTI (Asai et al. 2002; Shi et al. 2014). *PRI* gene expression, which

is a marker for salicylic acid (SA)-mediated resistance, was also examined in our study. Without *X. citri* challenge, the expression of *CsWRKY22*, *CsGST1*, or *CsPRI* was very low with no significant difference between negative controls or transgenic plants with low (FLS2-H12 and H14) or high (FLS2-H5 and H6) *NbFLS2* expression (Fig. 7A). These results suggest that ectopic expression of *NbFLS2* does not induce chronic expression of plant immunity and defense-associated genes. The expression of *CsGST1* was not significantly induced following *X. citri* infiltration in transgenic Hamlin compared with inoculated control plants or water-inoculation of plants (Fig. 7B). In contrast, the gene expression of *CsWRKY22* and *CsPRI* was significantly up-regulated at 24 hai in transgenic plants FLS2-H5 and H6 (Fig. 7C and D). No obvious gene upregulation was observed in control plants or transgenic plant FLS2-H9, which has low *NbFLS2* expression (Fig. 7). This indicates that *CsWRKY22* and *CsPRI* genes were upregulated only after *NbFLS2* perception of flagellin from *X. citri*.

DISCUSSION

Our earlier studies showed that flagellin from *X. citri*, '*Ca. L. asiaticus*', and '*Ca. L. solanacearum*' can induce PAMP responses and elevate the expression of genes associated with PTI by transient expression in *N. benthamiana*. In this study, we investigated whether heterologous expression of *NbFLS2* in citrus can increase resistance to canker. We expressed the flagellin receptor FLS2 from *N. benthamiana* in Hamlin sweet orange and Carrizo citrange. Transgenic citrus expressing *NbFLS2* showed increased ROS production that correlated with enhanced citrus canker resistance and defense-related gene activation, indicating that heterologous expression of *NbFLS2* is functional and can activate downstream citrus gene expression. We demonstrated that endogenous citrus *FLS2* genes have low expression in citrus and exhibit no or low levels of ROS production in response to the flg22_{Xcc}. We showed that the expression of *CsWRKY22* and *CsPRI* was upregulated only after *X. citri* infection in transgenic plants with high expression of *NbFLS2*. Our results suggest that heterologous overexpression of a functional FLS2 receptor followed by challenge with a compatible pathogen can activate the plant immunity pathway, which may lead to broad-spectrum disease resistance.

Fig. 2. Phylogenetic tree showing the relationship between FLS2 orthologs. The protein sequences included *Arabidopsis* AtFLS2 (GenBank accession number: NM_124003), tomato LeFLS2 (GenBank number: XP_004233092), *N. benthamiana* NbFLS2 (Niben.v4.2.Scfl4580), *Vitis vinifera* VvFLS2 (GenBank number: XP_002272319), potato StFLS2 (GenBank number: XP_6365570), poplar PtFLS2 (GenBank number: XP_002305701), rice OsFLS2 (GenBank number: CHA68341), citrus FLS2 homologs CsFLS2 (GenBank number: XP_006478775), and CsFLS2-L (orange 1.1 g000859m at the Joint Genome Institute Phytozome database). The MEGA6.06 program was used to construct the tree with the maximum likelihood method. The bootstrap consensus tree was inferred from 1,000 replicates and bootstrap values were indicated next to the branches. Fewer than 75% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1,182 positions in the final dataset.

One of the earliest cellular responses following successful pathogen recognition is a rapid and transient oxidative burst (Wojtaszek, 1997; Torres et al. 2006). We demonstrated increased ROS production in *NbFLS2* transgenic citrus, indicating that the FLS2 receptor has the ability to interact with flg22_{Xcc} and activate PTI responses. The nonhost defense response in the *A. thaliana* and *X. citri* interaction is apparent through ROS production and the expression of defense genes (An and Mou 2012). Recently, it was demonstrated that PTI was induced more significantly in the canker-resistant variety ‘Nagami’ kumquat compared with susceptible ‘Duncan’ grapefruit (Shi et al. 2014). ROS production was also shown to be correlated to the expression of defense-related genes and the reduction of canker development in the sour orange seedling screening (Y. Duan, *unpublished*). In our experiments, no significant ROS production was detected in canker-susceptible nontransgenic sweet orange Hamlin exposed to flg22_{Xcc}. Though some nontransgenic Carrizo seedlings showed slightly higher ROS production compared with nontransgenic Hamlin, it was relatively low compared with transgenic plants expressing *NbFLS2*. It is interesting that two ROS peaks were observed in transgenic Carrizo plant C57 (Fig. 4B). In *N. benthamiana*, typically only a single sharp ROS peak is induced by flg22 within 50 min after flg22

treatment; however, there is a report of a second broad weaker peak occurring 2 to 6 h after the first ROS peak returned to basal level (Segonzac et al. 2011). It has been suggested that a second ROS peak is associated with plant cell death when challenged with hypersensitive response-inducing pathogens (Ren et al. 2002). Further investigations are underway for this interesting transgenic Carrizo plant C57. Our results suggest that strengthening plant innate immune system via overexpressing a heterologous PAMP receptor is a promising method by which to enhance citrus disease resistance.

PTI can serve as the first layer of protection against non-pathogenic and pathogenic microbes. Plant reduced-function mutants associated with PAMP pathways, such as *A. thaliana* and *N. benthamiana* *FLS2* mutants, are more susceptible to nonadapted strains that normally don’t cause disease as well as to virulent *Pseudomonas syringae* (Zipfel et al. 2004). The PAMP receptor EFR from *A. thaliana* was expressed in both *N. benthamiana* and tomato plants and enhanced resistance to several plant pathogens from different genera. In addition, enhanced resistance has been previously reported for transgenic plants expressing genes associated with the plant defense system (Bouwmeester et al. 2014; Fagoaga et al. 2011). *Xa21* transgenic citrus plants showed enhanced resistance to citrus

Fig. 3. Comparison of reactive oxygen species (ROS) bursts in nontransgenic citrus seedlings and transgenic plants. Eight leaf discs from each seedling and transgenic plant were floated on water overnight in a 96-well plate at room temperature. Prior to ROS measurement, water was replaced with 100 μ l of assay solution. The assay was monitored about 40 min after addition of 100 μ l of assay solution (17 mM luminol, 1 μ M horseradish peroxidase, and 0.1 μ M flg22_{Xcc}) and was detected with chemiluminescence probe Luminol. **A**, Hamlin seedlings, **B**, Carrizo seedlings, **C**, transgenic Hamlin expressing *NbFLS2*, **D**, transgenic Carrizo expressing *NbFLS2*. *NbFLS2* polymerase chain reaction-negative plants were used as control plants in C and D. Bars represent peak luminescence reading across 40 min of each sample. Error bars represent standard deviation from eight leaf discs. CPS = count per second.

canker, indicating that the *Xa21* resistance gene from rice is functional in citrus (Mendes et al. 2010). Transgenic citrus expressing *A. thaliana* NPR1 protein, a SA signal transducer from *A. thaliana*, showed enhanced disease resistance to citrus canker (Zhang et al. 2010). Transgenic citrus expressing citrus MAPK increased ROS generation and resistance when challenged with *X. citri* (de Oliveira et al. 2013).

Our study demonstrates that endogenous *FLS2* homologs from citrus share low homology with *FLS2* from *N. benthamiana* and are normally expressed at low levels. Following flg22_{xcc} challenge, no significant ROS production was detected in canker-susceptible nontransgenic sweet orange (Fig. 3). This indicates that *FLS2* homologs from canker-susceptible varieties are insensitive to *X. citri* flagellin or that there is a weak interaction between the *FLS2* receptors and *X. citri* flagellin. Similar results were reported previously with reduced gene induction and weak ROS production in canker-susceptible citrus genotypes treated with flg22_{xcc} (Shi et al. 2014). It is not clear why there is a weak interaction between citrus *FLS2* homologs and *X. citri* flagellin. CsFLS2 shares a typical *FLS2* receptor structure with a ligand-binding ectodomain, a transmembrane domain, one domain containing 28 LRR, and contains the amino acids reported to be critical. However, CsFLS2-L contains several more LRR compared with reported functional *FLS2* orthologs and lacks several critical amino acids, including Ser-938, which are important for the PAMP-mediated immunity pathway (Cao et al. 2013). Though *CsFLS2* expression was detected by RT-qPCR in Hamlin, full-length *CsFLS2*, which was identified in NCBI Valencia sweet orange data, was not identified in the *C. sinensis* genome sequence from the JGI Phytome database. Further investigation is needed to clarify whether *CsFLS2* is present in all *Citrus sinensis* varieties.

Overexpression of *NbFLS2* in transgenic citrus increases canker resistance following spray inoculation but not infiltration. It is not surprising that no obvious difference was observed for canker development by infiltration, because studies have shown that PAMPs and *FLS2*-mediated immunity against pathogenic bacteria is associated, in part, with closure

of stomata (Melotto et al. 2006). Under natural conditions, *X. citri* infects citrus leaves through wounds or stomata. However, infiltration of bacteria with a syringe will bypass the first steps of the natural infection process. An *A. thaliana* *FLS2* mutant with reduced function did show more severe disease symptoms compared with wild-type plants after spraying *P. syringae* DC3000 onto leaf surfaces (Zipfel et al. 2004). Studies showed that canker resistance in the mandarin 'Okitsu' was bypassed using leaf infiltration (Favaro et al. 2014). Recently, flg22-induced oxidative burst and *FLS2* signaling activation of MPK3 and MPK6 were shown to be essential for control of stomatal closure in disease resistance (Mersmann et al. 2010; Montillet et al. 2013). Spray inoculation more closely resembles the natural infection process of *X. citri* through stomata, which allows flagellin or flg22_{xcc} to interact with *FLS2* receptors and activate PTI. Resistant and susceptible citrus genotypes have been reported to have differences in stomatal characteristics and stomatal response (apertures) following exposure to *X. citri* (Wang et al. 2011).

The constitutive overexpression of defense-related genes in plants can potentially lead to either necrosis, reduced growth, or both (Aldwinckle and Malnoy 2009). Transgenic *Arabidopsis* constitutively overexpressing *EDS5* showed activation of pathogen-related genes and enhanced virus disease resistance, however the transgenic plants were severely dwarfed (Ishihara et al. 2008). In addition, *Arabidopsis* overexpressing *NDR1* had enhanced bacterial disease resistance and also led to *PR1* constitutive expression, spontaneous lesions, and stunting (Coppinger et al. 2004). In our experiments, no developmental or growth defects were observed in transgenic citrus plants expressing *NbFLS2*. We showed no constitutive *CsPR1* gene expression in our transgenic plants expressing *NbFLS2*. Instead our results suggest that flagellin must be present to trigger *FLS2* to activate *CsWRKY22* and *CsPR1* gene expression (Fig. 7). We determined that the expression of the PTI marker gene *CsWRKY22* was highly induced 24 h after infiltration with *X. citri*. The induction of *CsWRKY22* was previously reported in canker-resistant cultivar 'Nagami' kumquat following

Fig. 4. Reactive oxygen species (ROS) assay for transgenic citrus expressing *NbFLS2*. Eight leaf discs from each seedling and transgenic plant were floated on water overnight in a 96-well plate at room temperature. Prior to ROS measurement, water was replaced with 100 μ l of assay solution. Assays were monitored at 40 or 60 min and were detected with chemiluminescence probe Luminol. **A**, Transgenic Hamlin plants H5 and H6 showed typical ROS response. No ROS response was observed with the flg22_{xcc}-treated *NbFLS2* polymerase chain reaction (PCR)-negative plant. **B**, Transgenic Carrizo plant C57 showed two peaks of ROS production and transgenic plant C22 showed only one peak. No ROS response was observed with the flg22_{xcc}-treated *NbFLS2* PCR-negative plant. Error bars represent the standard deviation from eight leaf discs. CPS: count per second.

flg22_{Xcc} treatment (Shi et al. 2014). In conclusion, we showed that heterologous expression of *NbFLS2* in citrus can increase ROS production and activate PTI and defense-associated gene expression. We demonstrated that the expression level of *NbFLS2* correlated with canker suppression as well as ROS production level. Our results indicate that a functional flagellin perception system exists in citrus. It remains of interest to investigate whether citrus *FLS2* orthologs interact with flagellin of *X. citri* or '*Ca. Liberibacter asiaticus*' and whether over-expression of citrus *FLS2* can increase disease resistance. Furthermore, transgenic citrus trees expressing *NbFLS2* are under

further investigation regarding their resistance to citrus greening disease.

MATERIALS AND METHODS

Citrus seed preparation and germination.

Seeds were extracted from mature fruits of Hamlin sweet orange [*C. sinensis* (L.) Osbeck] and Carrizo citrange [*C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] and were treated with pectinase overnight. The seed coats were removed and the surface of seeds were sterilized by soaking in ETOH. The

Fig. 5. Comparison of reactive oxygen species (ROS) production, citrus canker symptoms, and canker lesions in leaves of a *NbFLS2* polymerase chain reaction–negative plant as control and transgenic plants. **A**, ROS production in transgenic Hamlin and control; **B**, ROS production in transgenic Carrizo and control. **C**, Canker symptoms in transgenic Hamlin and control; **D**, canker symptoms in transgenic Carrizo and control. **E**, Canker lesion numbers in transgenic Hamlin and control; **F**, canker lesion numbers in transgenic Carrizo and control. *Xanthomonas citri* was applied by spray inoculation and was evaluated at 30 days postinoculation. Bars represent means of three inoculated leaves. Two replicated plants of each transgenic parent plant were tested with similar results.

seeds were then treated with sodium hypochlorite solution (10%) for 10 min, followed by rinses with sterile distilled water. The seeds were planted in Magenta jars containing Murashige Skoog (MS) solid medium and were incubated in the dark at 27°C for 2 to 3 weeks. Germinated seedlings with a height of 12 to 15 cm were transferred to a 16-h photo-period growth chamber for 10 days. After this period, the epicotyls of the seedlings were cut for *Agrobacterium*-mediated transformation experiments.

Gene cloning and vector construction.

RNA was isolated from leaves of *N. benthamiana* and cDNA was obtained by reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Primers for *NbFLS2* cDNA amplification were designed containing *Sma*I and *Spe*I restriction sites (Table 1). The PCR reactions were performed in a thermal cycler under the following conditions: 3 min at 95°C for the first denaturation, followed by 26 cycles of 30 s at 95°C, 45 s at 53°C, and 4 min at 68°C, plus a final extension of 10 min at 68°C.

Fig. 6. Canker lesions incited by *Xanthomonas citri* spray inoculation in transgenic plants and control plants. Lesions were surveyed 25 days after inoculation. Two replicated plants from each transgenic plant were sprayed at each time point and the experiment was repeated three times. Statistical analysis was performed with one-way analysis of variance and Tukey's posttest with a P value < 0.05 . Within each group (A or B), columns with different letters are significantly different at the $P < 0.05$ level. Each column represents the mean and error bars are the standard deviation of the mean ($n = 6$). **A**, Nontransgenic control versus transgenic Hamlin plants expressing *NbFLS2*. **B**, Nontransgenic control versus transgenic Carrizo plants expressing *NbFLS2*.

Fig. 7. Gene expression in transgenic Hamlin plants expressing *NbFLS2* and control plants. **A**, Comparison of the expression of *CsPR1*, *CsWRKY22*, and *CsGST1* in transgenic Hamlin expressing *NbFLS2* and control plants without *Xanthomonas citri* inoculation. **B**, **C**, and **D**, *CsGST1*, *CsWRKY22*, and *CsPR1* expression in transgenic Hamlin FLS2-H5, H6, H9, and control plants without *X. citri* inoculation, 6 and 24 h following water or *X. citri* infiltration. Bars represent relative mRNA fold change of *CsPR1*, *CsWRKY22*, and *CsGST1* in transgenic plants and control plants. The samples were normalized against the citrus *GAPC2* gene. Data are shown as average fold induction compared with control plants from three independent experiments.

PCR products were purified and were digested with *Sma*I and *Spe*I and were ligated into pBinPlus/ARS containing a double 35S promoter from *Cauliflower mosaic virus* and a Nos terminator. The plasmid was sequenced, confirmed, and designated as pBinARS-*NbFLS2*. *Agrobacterium tumefaciens* EHA105 was transformed with pBinARS-*NbFLS2* by electroporation. Transformed *A. tumefaciens* EHA105 cells were confirmed by PCR and were used for citrus transformation.

Genetic transformation and plant regeneration.

Epicotyl segments (0.8 to 1.0 cm) were cut from in vitro germinated seedlings and were incubated with *Agrobacterium* carrying pBinARS-*NbFLS2* (optical density at 600 nm (OD₆₀₀) of about 0.7 to 0.8) for 20 min, were blotted dry, and were transferred to coculture medium (CM1) supplemented with benzylaminopurine (BAP) at 1.0 mg per liter for 3 days (24°C, in the dark). After the coculture period, explants were transferred to MSB1 selection medium supplemented with BAP, kanamycin, and cefotaxime (1.0, 100, and 500 mg per liter, respectively). Cultures were incubated in the dark (27°C) for 2 to 3 weeks and were then transferred to a 16-h photoperiod at 27 ± 1°C.

Kanamycin-resistant shoots were carefully excised from the explants and roots were induced on MS medium with the addition of 1 µM spermidine (Mendes et al. 2011). Well-developed plantlets were transferred to container pots with soil-less mix and were kept in a greenhouse.

Molecular analysis of transformed plants.

Genomic DNA was isolated from leaves of independent transgenic plants and control plants by Qiagen kit and were evaluated by PCR. Primers were designed based on the sequence of *NbFLS2* and Nos terminator regions (Table 1). Transgenic plants containing the target gene were used for further analysis. RNA was extracted using TRIzol reagent according to the manufacture's instruction (Sigma-Aldrich, St. Louis). Total RNA was quantified using the Nanodrop (Thermo Fisher Scientific, Wilmington, DE, U.S.A.) and was treated with RQ1 RNase-free DNase (Promega Corp, Madison, WI, U.S.A.). DNase-treated RNA (approximately 1.5 µg) from control and transgenic leaves was used to synthesize first-strand cDNA with 0.5 µg of oligo (dT) primer and 1 µl of SuperScript III reverse transcriptase in a 20-µl reaction (Invitrogen). A negative control without the reverse transcription was performed to verify the absence of genomic DNA contamination.

RT-qPCR was performed with Bright Green (Sigma-Aldrich) in triplicate, using an ABI7500 thermal cycler (Applied Biosystems, Foster City, CA, U.S.A.). The 25-µl amplification reactions contained the following: 12.5 µl of Bright Green PCR master mix, 250 nM of each forward and reverse primer, and 2.0 µl of 1:10 diluted cDNA template. The following protocol was used: 95°C for 2 min, 40 cycles of 30 s for denaturation at 95°C, and 30 s for extension at 60°C. Primers for *NbFLS2*, *CsFLS2*, and *CsFLS2-L* are listed in Table 1. The citrus gene glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPC2*) was amplified and was used to normalize the values as an internal control (Mafra et al. 2012). To compare transcript levels, a ratio of relative gene expression is calculated from the cycle threshold ($2^{\Delta Ct}$) values of a sample versus a reference sample that had the lowest $2^{\Delta Ct}$ in the tested samples, $\Delta Ct = Ct$ (internal control) – Ct (target gene). The qPCR reactions were set up in triplicate and were repeated twice with similar results.

Sequence alignment and phylogenetic analysis.

Protein sequence alignment analysis was performed using SV CLC Sequence program (Qiagen, Boston). Phylogenetic trees were constructed using the MEGA6 program (Tamura et al.

2013) with the maximum likelihood method (Whelan and Goldman, 2001) and bootstrap value 1,000.

ROS production assay.

The flg22_{Xcc} peptide (QRLSSGLRINSAKDDAAGLAIS) from *X. citri* was synthesized by GenScript (Piscataway, NJ, U.S.A.). Young tender citrus leaves from transgenic plants and nontransgenic control plants were used for ROS assays. Eight leaf discs from each sample were taken, using a 0.5 cm diameter cork borer, and were floated on water overnight in a 96-well plate at room temperature. Prior to ROS measurement, water was replaced with 100 µl of assay solution (17 mM luminol, 1 µM horseradish peroxidase, and 0.1 µM flg22_{Xcc}) (Hao et al. 2014). Luminescence was measured using a LUMistar microplate luminometer (BMG, Cary, NC, U.S.A.). Each plate was measured over a period of 40 or 60 min after addition of assay solution. Similarly, leaf discs were taken from nontransgenic Hamlin and Carrizo seedlings and the ROS assay was performed. The assay was repeated twice. Controls lacking flg22_{Xcc} were included for each plant. Data were analyzed using Optima software (BMG, Cary, NC, U.S.A.).

Evaluation of resistance to *X. citri* in transgenic plants.

Transgenic plants and nontransgenic control plants of Hamlin and Carrizo were selected for disease resistance evaluation. The infiltration experiments were performed on independent transgenic plants and nontransgenic control plants. *X. citri* was grown overnight in Luria Bertani liquid and were centrifuged and the pellet was diluted, to an OD₆₀₀ = 0.3, in autoclaved tap water. Serial dilution plating was used to determine bacterial population. Three young fully expanded leaves of each plant were infiltrated with *X. citri* at 10⁴, 10⁵, 10⁶, and 10⁷ CFU per milliliter. Plants were also spray-inoculated with a 10⁶ CFU per milliliter suspension of *X. citri* with the addition of 0.02% silwet-77. Three young fully expanded leaves were spray-inoculated on each plant. Disease severity was evaluated frequently after inoculation. Images were taken 4 weeks after spray inoculation. Three independent experiments were performed with similar results.

Bacterial growth determined by qPCR.

Bacterial growth in leaves was assessed by qPCR with the DNA isolated from leaf discs of infiltrated leaves in the zone of inoculation at 2 h and 3, 6, 9, and 12 dai. Fresh tissues were homogenized for genomic DNA extractions by Qiagen kit and were used for qPCR analyses. The presence of the *pthA* gene was determined using gene-specific primers VM3 and VM4, designed for *X. citri* quantification (McCollum et al. 2011). Primers were added at 0.5 µM in a 25-µl reaction with 12.5 µl of 2× Bright Green master mix reagent (Sigma-Aldrich) and 2 µl of DNA template (about 100 ng). Real time PCR was performed as described above. Based on a standard curve, the formula Copy Number (X_{cc} , *pthA*) = $10^{(38.3 - ct)/3.56}$ was used for quantification of *X. citri* (Stover and McCollum 2011). At each time point, samples were determined from three technical replicates and each experiment was done in duplicate. Control sampling was performed on leaf discs taken from non-transformed plants.

Gene expression analysis induced by *X. citri*.

Gene expression induced by *X. citri* was evaluated in leaves of noninoculated, water-infiltrated, and *X. citri*-infiltrated plants at 6 and 24 h after infiltration. Primer pairs are listed in Table 1. Primers for *CsGST1*, *CsWRKY22*, and *CsPR1* were designed according to *Citrus sinensis* genomic data from NCBI, with Loc102614737, Loc102612567, and Loc102623146 respectively. *CsGST1* and *CsWRKY22* were examined because both

genes were induced in canker-resistant citrus varieties by *X. citri* flg22 (Shi et al. 2014). In addition, *CsPRI* was upregulated in 'Ponkan' mandarin by *Xylella fastidiosa* infection (Rodrigues et al. 2013). The pellet of *X. citri* was suspended in sterile tap water and was adjusted to 3×10^5 CFU/ml for gene expression analysis. The bacterial suspension was infiltrated into three whole leaves of each transgenic and control plant by infiltration of the abaxial leaf surface with a needleless syringe. The leaves of transgenic and control plants were also mock-inoculated with sterile tap water. Three leaf discs were collected from infiltration zones of each leaf, using a round punch (6 mm in diameter), as independent biological replicates. RNA isolation, cDNA synthesis, and RT-qPCR were performed as described above. The gene expression level was normalized against the internal control *CsGAPC2*. The relative mRNA expression levels were normalized with noninoculated plant samples.

ACKNOWLEDGMENTS

We thank E. Cochrane, Y. Avila, and M. Sewell for their excellent technical assistance. Funding was provided by the Citrus Research and Development Foundation. We also thank G. Martin from Cornell University for *N. benthamiana* FLS2 information. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Joint Genome Institute (JGI) Phytozome database:
<http://phytozome.jgi.doe.gov/pz/portal.html>