Analysis of Defense-Related Gene Expression in Citrus Hybrids Infected by *Xylella fastidiosa*

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ABSTRACT

Resistance to *Xylella fastidiosa* was evaluated in 264 hybrids of crosses between Murcott tangor (*Citrus reticulata* × *Citrus sinensis*) and Pera sweet orange (*C. sinensis*) under field conditions. Uninfected hybrids were grafted with buds collected from Pera sweet orange plants infected with *X. fastidiosa*, forming a plant with two scions (i.e., hybrid branches and Pera sweet orange branches). From these plants, we chose 10 genotypes with three biological replicates. We evaluated gene expression, bacterial multiplication, and citrus variegated chlorosis (CVC) symptom development in both scions. *X. fastidiosa* was not detected in most hybrid scions and none showed disease symptoms. In contrast, all Pera sweet orange scions were infected with *X. fastidiosa* and expressed symptoms of CVC. We quantified the expression of 12 defense-related genes by qPCR comparing resistant to susceptible scions. We suggest that some of these genes are involved in resistance of the hybrids to *X. fastidiosa*, since their

expression was significantly higher in the resistant hybrid scions than in tolerant hybrids and scions originated from CVC symptomatic Pera sweet orange buds. However, we note that these data should be interpreted carefully, as the plant genotypes tested are related but necessarily distinct (hybrids of *C. reticulata* and *C. sinensis*, in relation to a *C. sinensis* control). A principal component analysis revealed a relationship between the expression of these genes and hybrid scions, and between scions that originated from infected buds and the presence of the bacteria and plant symptoms. Multiyear field trials are necessary to develop plant resistance to *X. fastidiosa*. While the experimental design used here had limitations, it allowed us to identify a set of genes potentially involved in *Citrus* sp. resistance to this pathogen. Future work on the role of these genes in plant defenses to *X. fastidiosa* infection is necessary to confirm their importance in the displayed resistance phenotype.

Around 25% of Brazil's gross domestic product is based on agriculture, of which the citrus industry, especially the production of sweet orange for orange juice, is one of the leading sectors. Indeed, Brazil is the world's largest exporter of orange juice, and around 70% of Brazil's sweet orange crop is cultivated in São Paulo State. However, in the last 20 years, phytosanitary problems have increased the cost of production, resulting in a decrease of cultivated area, especially that managed by smaller growers. In the last decade, 40 million citrus plants were eradicated in Brazil due to pests and diseases (Neves et al. 2010).

Among the various diseases affecting citrus in Brazil, citrus variegated chlorosis (CVC), caused by the xylem-limited bacterium *Xylella fastidiosa* subsp. *pauca* (Purcell 2013), is one of the most damaging. CVC is transmitted by leafhopper vectors in the family Cicadellidae, and also by infected budwood (Almeida and Nunney 2015). The disease is characterized by obstruction of xylem of the plant, leading to insufficient translocation of water in the vessels colonized *X. fastidiosa* bacteria (Pria et al. 2003).

X. fastidiosa has a very wide host range, and the various subspecies of this pathogen can cause several economically important diseases such as Pierce's disease of grape, plum leaf scald, phony peach disease, CVC, and olive quick decline. In Brazil, CVC, caused by *X. fastidiosa* subsp. *pauca*, was first described in 1987 (Rossetti et al. 1990). The use of resistant plants is the most appropriate solution for the control of CVC (Purcell 1994). In citrus, some sources of resistance to CVC and *X. fastidiosa* have been reported, but all the commercially cultivated sweet orange (*C. sinensis* L. Osb.) varieties are susceptible to the disease (Laranjeira et al. 1998). The cultivars Navelina ISA 315, Navelina SRA 332, and Newhall navel SRA 343 were asymptomatic hosts of *X. fastidiosa* (Souza et al. 2006). In agreement with previous information, Fadel et al. (2014) showed that Navelina ISA 315 is resistant to CVC, as no symptoms and low bacterial concentrations were found. Some mandarins (*C. reticulata*) are considered resistant because they do not allow multiplication of the bacterium. In contrast, tolerant plants show no symptoms, but *X. fastidiosa* can be detected within the xylem (Laranjeira et al. 1998; Rodrigues et al. 2013). Laranjeira et al. (1998) reported that several genotypes of mandarin (*C. reticulata*), lime (*C. aurantiifolia*), lemon (*C. limon*), grapefruit (*C. paradisi*), pummelo (*C. grandis*), and tangor (*C. sinensis* × *C. reticulata*) were negative for *X. fastidiosa* when evaluated under field conditions in areas containing high disease pressure. Coletta-Filho et al. (2007) reported results obtained for citrus hybrids of the same progeny of the present work. The results of 20 hybrids and their parents sweet orange cultivar Pera, susceptible, and tangor cultivar Murcott, resistant, showed a complete spectrum of response to CVC disease. Resistant, tolerant, and susceptible reactions were observed for the hybrids, demonstrating the existence of genetic variation for CVC resistance.

To improve CVC management, a better understanding of the complex interactions that exist among the host, pathogen, and

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vector and the possible mechanisms involved in disease resistance is necessary. Previous works (De Souza et al. 2004, 2005, 2007, 2009; Rodrigues et al. 2013) have investigated these interactions, but not in field conditions. Thus, the objective of this study was to compare the differential expression of genes in resistant/tolerant Murcott tangor (*C. reticulata* × *C. sinensis*) × Pera sweet orange (*C. sinensis*) hybrids and susceptible Pera sweet orange scions, cultivated under field conditions over 6 years.

MATERIALS AND METHODS

Biological assay. Buds of 264 hybrids derived from crosses between Murcott tangor and Pera sweet orange and their parents were grafted onto Rangpur lime (*C. limonia*) in the greenhouse. After 1 year, plants were inoculated with two or three axillary buds taken from Pera sweet orange trees with symptoms of CVC. Six months after inoculation, the composite trees were transplanted to the field and allowed to develop from 2007 to 2013 at the Paulista Agency of Agribusiness Technology (APTA) in the municipality of Colina, São Paulo State, a region endemic with CVC. To reinforce infection, buds used as inoculum were left to develop, forming a plant with two scions (i.e., one branch of hybrid and another branch of Pera sweet orange used as inoculum source) (Supplementary Fig. S1). The experimental design was completely randomized including four biological replicates of each genotype.

Disease assessment and detection of *X. fastidiosa*. CVC severity was assessed by visual observation of leaf symptoms once a year between 2008 and 2013 using a diagrammatic scale. The scale included six levels of severity (3, 6, 15, 25, 35, and 56% area with chlorosis) (Amorim et al. 1993) and it was applied to five leaves per symptomatic plant.

Bacterial populations were quantified by qPCR with *X. fastidiosa*-specific primers according to Oliveira et al. (2002). Six leaves of each hybrid branch and each Pera sweet orange branch that originated from buds infected with *X. fastidiosa* were collected and total DNA was extracted from 100 mg of tissue from the central veins of fresh and clean leaves using the method of Doyle and Doyle (1990). DNA concentration was determined with a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). qPCR was performed using fast TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA). The reaction consisted of 1.5 µl of labeled probe to *X. fastidiosa* at a concentration of 6,000 pmol and 1.5 µl of the primers CVC-1 and CCSM-1 as described by Oliveira et al. (2002), and 2 µl

of DNA diluted to 50 ng/µl. Amplifications were carried out with three technical replicates for each sample, including negative controls. An ABI PRISM 7500 SDS (Applied Biosystems) was programmed as follows: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s, and 60°C for 1 min. Quantification was assessed based on the number of amplification cycles needed to reach a common fixed threshold (cycle threshold [Ct]) in the exponential phase of PCR.

All qPCR results reported were used to estimate the number of cells of *X. fastidiosa* in each sample from the standard curve (Oliveira et al. 2002). The standard curve was previously developed based on five points (serial dilutions of 1×10^3 to 1×10^8) with defined concentrations of *X. fastidiosa*, resulting in the equation $y = -3,3483x + 38,196$ ($r^2 = 0.99$). Negative (no DNA template) and positive (DNA of *X. fastidiosa*) controls were included in all experiments.

Gene expression analysis. We selected 10 genotypes from the biological assay, with three biological replicates from a plant with two scions (i.e., one hybrid branch and one Pera sweet orange branch that was the inoculum source). We evaluated gene expression, bacterial multiplication, and CVC symptom development in both scions. Total RNA was isolated from leaves collected separately from the hybrid and sweet orange branches of each replicate in 2012. For RNA isolation TRIZOL (Invitrogen, Carlsbad, CA) and purified with RNeasy plant mini kits (Qiagen, Hilden, Germany) according to manufacturer's recommendations. To avoid contamination by genomic DNA, all samples were treated with RNase-free DNase (Qiagen). Total RNA was quantified by spectrophotometry (Nanodrop 8000 Spectrophotometer (Thermo Scientific), and the integrity of the RNA was evaluated by electrophoresis on denaturing agarose gels that contained formaldehyde. cDNA synthesis was performed using the Revertaid H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA) using random primers (Fermentas) from 1 µg of total RNA, according to the manufacturer's instructions. The cDNAs were used in duplicate in the following reaction: 3 µl of cDNA diluted (1:25), 2 µl of each primer (forward and reverse), 10 µl of Promega GoTaq (Promega, Madison, WI) and 5 µl of Milli-Q water. The reactions were incubated at 50°C for 2 min, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The amplifications were performed in an ABI PRISM 7500 Fast SDS version 1.4 (Applied Biosystems).

qPCR was performed to evaluate the expression of defense-related genes selected in a microarray assay (Supplementary File S1) focused on the differential expression of UniGene transcripts

TABLE 1. Defense-related genes and associated qPCR primer sequences used in gene expression comparisons between resistant and susceptible hybrids following challenge with *Xylella fastidiosa* in a field trial near Colina, São Paulo, Brazil

Gene symbol	Gene id <i>Citrus sinensis</i> ^a	Gene description	Forward (5'–3')	Reverse (5'–3')
b-ZIP	Cs3g23480	Basic leucine zipper domain, ABA responsive element binding factor	GTAATGATGATTGGTTGGAGG	TTTCTCCCATATTGAGGGAC
Thaumatin	Cs3g08300	Pathogenesis-related group 5 protein, osmotin, thaumatin-like protein	TAGGACCAATTCTGTCTCAC	ATATCTCATTGGCTTCCCT
RGA2	Cs9g13300	Disease resistance protein RGA2, defense response	AGTAGAAAGTGATACGGATGG	ATCAAGGAAAGAAAGACGTG
DRP	Cs5g22440	Disease resistance protein (TIR-NBS-LRR class), defense response	GAATGAGTTGCCTACCTCC	TGTCATGAATCAAGTTGGG
Chitinase	orange1.1t01780	Endochitinase, carbohydrate metabolic process, pathogen resistance	ACAGAATTGTGGAAGCGG	AGCAAGTCTTCAAACATCTCC
LRR-RK	Cs1g02030	Protein kinase activity, protein phosphorylation	CAGGCATCGTAATCTTGTC	CCAGAATCCAAATCTCCCA
STK	Cs4g01430	Catalytic activity, protein serine/threonine kinase activity, carbohydrate metabolic process	ATGCAACAATTACACAGCC	ACCTTAATGATTGAGTGCCA
FLS2	Cs2g15620	LRR receptor-like serine/threonine-protein kinase FLS2, protein binding	GGAGCTTAAGAAATCTAGAGGG	GTGTTATCCTCACAACAGTC
IFR	Cs5g17380	Nucleotide binding, NAD dependent epimerase	TATCCATTATACACAGCGCA	CAAGAAGTTGATCGATTGTGG
P450	Cs4g13780	Cytochrome P450, Interacting selectively and noncovalently with iron (Fe) ions	TCTCGATCTTCTTACTGTCC	ACTGAGTAAACAGACACTCAC
Ankyrin	Cs1g26140	Ankyrin repeat domain-containing protein, mediate protein-protein interactions	AGTTTGACCAGAGAAAGAGAC	TTTGTTAGCAATCAACGCAG
GST	Cs5g32800	Glutathione S-transferase, protein binding	CTGATCTGAGTCACCTTCCA	GGCCTGTTGCTAATATCATCC

^a *Citrus sinensis* transcript identification numbers available in the database: <http://citrus.hzau.edu.cn/orange/index.php>.

from the comparison between plants that were resistant and plants that were susceptible to *X. fastidiosa* (Diogo et al. 2011) (Supplementary Fig. S2). Specifically, 12 genes were selected based on their functions, which included genes involved in defense responses (Table 1). Quantitative qPCR was performed using Power SYBR Green PCR Master Mix reagent (Applied Biosystems). The reaction consisted of 3.0 µl of cDNA and 120 nM of each gene-specific primer in a final volume of 20 µl. Amplification was carried out with three technical replicates for each sample, including negative controls. An ABI PRISM 7500 SDS (Applied Biosystems) was programmed with the following thermal cycles: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s, and 60°C for 1 min.

For relative quantification, the $2^{-\Delta\Delta CT}$ method was applied (Livak and Schmittgen 2001). Gene expression in hybrids branches and Pera sweet orange branches was expressed as fold changes in relation to an uninfected plant of Pera sweet orange. β -Tubulin, ubiquitin, and GAPC2 were used as reference genes. Fold change was used to indicate substantial differences (greater than 2.0-fold up or –2.0-fold down).

Statistical analysis. Analysis of the overall gene expression profile of the experiment was performed by grouping the candidate genes with a hierarchical clustering test, using the 10 resistant/tolerant hybrids (composed of branches of hybrids and Pera sweet orange) ordered by the number of bacteria found in the plant material, with the program MeV (Multi Experiment Viewer) v. 4.9 (www.tm4.org). The results were visualized with a Heatmap.

Pearson's correlation and principal component analyses were performed to determine the relationships among gene expression, bacterial multiplication, and the presence of symptoms in both the hybrid scions and in the scions that originated from buds of CVC symptomatic Pera sweet orange trees. The analyses were carried out in the program XLStat (Addinsoft 2017; https://www.xlstat.com/en/solutions/base#tutorials).

RESULTS

Field evaluation for CVC resistance. Based on evaluations performed during 6 years after the establishment of the plants in the field, 84 hybrids (31.8%) were symptomatic for CVC and qPCR-positive for *X. fastidiosa* among the 264 hybrids evaluated. Most of these plants had disease severity values that were intermediate between Murcott tangor (0%) and Pera sweet orange (average of 4.25%) (Fig. 1). Fifty-five hybrids showed no symptoms and were negative by qPCR, whereas 125 had no symptoms but were positive by qPCR. All branches that originated from buds of Pera sweet orange infected with *X. fastidiosa* showed symptoms of CVC.

Fig. 1. Frequency distribution of the percentage of leaf area with symptoms of citrus variegated chlorosis in 84 hybrids and parents of Murcott tangor and Pera sweet orange in a field trial near Colina, São Paulo, Brazil.

Ten hybrids that showed no visual symptoms of CVC were selected for more detailed analyses; however, in three of these hybrids (H1, H2, and H6), *X. fastidiosa* was detected by qPCR (Table 2). We qualified these three hybrids as tolerant because they did not show symptoms of CVC but they allowed the multiplication of the bacterium in the plant. Hybrids H3, H4, H5, H7, H8, H9, and H10 were qualified as resistant. Two of these, hybrids H5 and H8 (Supplementary Fig. S3), have great potential for juice and fresh fruit markets. All Pera sweet orange branches taken from these same 10 composite plants showed symptoms of CVC and bacterial growth was detected by qPCR (Table 2).

Gene expression analysis. Gene expression in hybrid branches and symptomatic Pera sweet orange branches was expressed as fold changes in relation to an uninfected plant of Pera sweet orange, whereby fold changes greater than 2.0-fold up or –2.0-fold down were considered substantial. Based on the heatmap used to visualize the gene expression patterns (Fig. 2), it is apparent that most of the genes were upregulated in the hybrids, suggesting that they may be related to the plant's defense response. In contrast, in the corresponding branches of Pera sweet orange (I2, I6, I11, I10, I3, I9, I8, I7), most genes were downregulated. Hybrids H4 and H8 had most of the upregulated genes and they are resistant to the disease based on the absence of *X. fastidiosa* in the qPCR assay. The tolerant hybrids H1, H2, and H6, which were asymptomatic for CVC but positive by qPCR, showed lower levels of expression of the target genes, indicating again that these genes may be involved in resistance to infection by *X. fastidiosa*.

TABLE 2. Quantification of *Xylella fastidiosa* by real-time quantitative PCR in 10 citrus plants having asymptomatic hybrid branches and symptomatic Pera sweet orange branches in a field trial near Colina, São Paulo, Brazil

Plant	<i>X. fastidiosa</i> population (mg/µl)	
	Pera sweet orange branch (inoculum buds)	Hybrid branch
1	2.5597	0.6339
2	1.9236	1.6930
3	3.0210	0
4	0.8921	0
5	2.3770	0
6	2.2289	0.5925
7	3.3207	0
8	3.2018	0
9	3.0998	0
10	3.0074	0

Fig. 2. Heatmap of fold changes used to visualize substantial differences (greater than 2.0-fold up or –2.0-fold down) in gene expression in resistant Murcott tangor hybrids and susceptible Pera sweet orange following challenge with *Xylella fastidiosa* in a field trial near Colina, São Paulo, Brazil. Gene symbols are defined in Table 1. H1 to H10 are the hybrid branches and I1 to I10 are Pera sweet orange branches from the same tree (Table 2).

Correlation and principal component analysis. Across the 10 genotypes, Pearson's correlation coefficients were calculated to determine the relationship between expression of each of the twelve target genes and symptom severity (%) or bacterial population (mg/μl) (Table 3). Bacterial populations were significantly and negatively correlated with the expression levels of the genes b-ZIP, thaumatin, RGA2, DRP, chitinase, LRR-RK, FLS2, and IFR. Symptom severity also was significantly and negatively correlated with the expression levels of b-ZIP, RGA2, DRP, chitinase, LRR-RK, FLS2, and IFR. Conversely, the expression levels of STK, P450, ankyrin, and GST did not show significant correlations (Table 3).

Principal component analysis of gene expression levels, bacterial population, and symptom severity (Fig. 3) revealed that the first two principal components explained 68.7% of the total variation (PC1 = 57.8% and PC2 = 10.9%). PC1 increased with increasing expression levels of all genes, while it decreased with increasing bacterial population and symptom severity, suggesting that branches of plants with high bacterial and symptom levels would tend to have lower gene expression levels and vice versa. PC2 increased with increasing expression levels of the genes STK, thaumatin, P450, ankyrin and GST, while it decreased with increasing expression of RGA2, b-ZIP, DRP, IFR, and LRR-RK in leaf tissue. The principal components plot generated using the loadings of the 10 plants on PC1 and PC2 clearly separated Pera sweet orange branches from

those of the hybrids scions, further supporting the consistent differences between the two groups of genotypes (Fig. 3).

DISCUSSION

CVC is one of the most important diseases for orange growers in Brazil. To integrate breeding and genomics programs to provide resistance against this pathogen, genes involved in disease responses need to be identified. In this study, we proposed that genes differentially expressed between the resistant or tolerant hybrids of Murcott tangor × Pera sweet orange and the susceptible Pera sweet orange may provide key candidates for identifying transcripts involved in disease resistance. First, we evaluated 264 hybrids derived from crosses between Murcott tangor and Pera sweet orange in the field over a period of 6 years. As expected, we found a wide range of responses including susceptible (visual symptoms and positive by qPCR), tolerant (asymptomatic but positive by PCR), and resistant (asymptomatic and negative by qPCR). These results are consistent with previous observations of different *Citrus* species under conditions of natural infection, where some species such as sweet oranges are susceptible and others such as mandarins are considered resistant because they do not allow multiplication of the bacteria; yet others are considered tolerant because they remain asymptomatic but *X. fastidiosa* can be detected in plant tissue (Coletta-Filho et al. 2007; Laranjeira et al. 1998; Rodrigues et al. 2013).

TABLE 3. Pearson's correlation coefficient between the expression of 12 putative defense genes and *Xylella fastidiosa* population (mg/μl) or severity of citrus variegated chlorosis (%) across 10 citrus plants having asymptomatic Murcott tangor hybrid branches and symptomatic Pera sweet orange branches in a field trial near Colina, São Paulo, Brazil

Traits	Coefficients of correlation ^a											
	b-ZIP	Thau	RGA2	DRP	Chit	LRR-RK	STK	FLS2	IFR	P450	Anky	GST
Bac.Qua.	-0.732*	-0.451*	-0.766*	-0.502*	-0.554*	-0.708*	-0.316 NS	-0.617*	-0.689*	-0.255 NS	-0.418 NS	-0.389 NS
Symptom	-0.694*	-0.401 NS	-0.665*	-0.567*	-0.473*	-0.645*	-0.303 NS	-0.577*	-0.643*	-0.217 NS	-0.387 NS	-0.355 NS

^a Asterisk indicates that correlation was significantly ($P \leq 0.05$).

Fig. 3. Principal component analysis scatter plots based on gene expression data, *Xylella fastidiosa* population, and citrus variegated chlorosis symptom severity across 10 citrus plants having asymptomatic Murcott tangor hybrid branches (H1 to H10) and symptomatic Pera sweet orange branches (I1 to I10) in a field trial near Colina, São Paulo, Brazil. **A**, Relationship of the two principal components (PC1 and PC2) to the three original variables. **B**, Discrimination between the asymptomatic hybrid branches and the branches of symptomatic Pera sweet orange based on PC1 and PC2.

According to Coletta Filho et al. (2007) the symptoms of CVC are associated with the blockage of xylem vessels by *X. fastidiosa* biofilm, leading to increased water stress and decreased nutrient supply in the diseased plant. However, the resistance of mandarin is not related to the xylem anatomy (number and diameter of vessels), suggesting that resistance is instead caused by active defense responses (Niza et al. 2015).

The listing of upregulated genes found in our study includes those coding for disease resistance proteins (RGA2 and DRP), protein kinase activity (LRR-RK and FLS2), a nucleotide binding protein (IFR2), and a transcription factor (b-Zip). All these genes are related to the initial response of plants to pathogen infection. bZIP transcription factors regulate many processes in plants, such as stress response and pathogen defense. Isoflavone reductase (IFR) is an enzyme involved in the biosynthetic pathway of isoflavonoid phytoalexin in plants and have crucial role in plant response to biotic and abiotic stresses. For example, Cheng et al. (2015) reported that the overexpression of isoflavone reductase in soybean enhances resistance to *Phytophthora sojae*. In rice, the overexpression of an isoflavone reductase-like gene (OsIRL) confers tolerance to reactive oxygen species (Kim et al. 2010). Here, we also observed that plants with high expression of this gene were more resistant to *X. fastidiosa*.

Another upregulated gene in the resistant hybrids was RGA2, a disease resistance protein that belongs to the NB-LRR family which restricts pathogen growth in plants (Song et al. 2003). In resistant citrus hybrids, we observed a complete restriction of pathogen growth, suggesting a possible role of this gene during the plant's interaction with the pathogen. FLS2 encodes an LRR-RK (leucine-rich repeat receptor kinase) and is correlated with the flagellin response in *Arabidopsis*. The functional kinase activity is required for activation of signal transduction, for ligand binding and for pathogen recognition (Gomez-Gomez et al. 2001). Although *X. fastidiosa* is nonflagellate, the LRR-RK found here could be correlated with another protein recognized by the citrus plant during the plant-pathogen interaction (Danna et al. 2011).

The present study focused on resistant and tolerant reactions observed in 10 hybrids between Murcott tangor and Pera sweet orange maintained under field conditions for a period of 6 years. The results demonstrated the existence of segregation of CVC resistance to the hybrid progeny and the possible inheritance of CVC resistance from Murcott tangor parent to the hybrids with fruit traits that have potential for the juice industry or for the fresh fruit market. Most of the upregulated genes in resistant plants were involved in processes of pathogen recognition and activation of defense responses, suggesting that these plants have active defense mechanisms as opposed to preformed defenses related, for example, to xylem anatomy.

While our screening allowed for the identification of defense genes correlated with CVC symptoms and *X. fastidiosa* populations in plants, there were limitations to this study. First, a large number of hybrids were tested with high disease pressure under field conditions for several years, in addition to graft-mediated pathogen infection. From a practical perspective, it would not have been feasible to establish adequate negative (i.e., uninfected) controls in these conditions, and therefore that was not attempted here. Similarly, because hybrids are by definition genetically distinct from each other (segregating F1), gene expression comparisons among hybrids should be interpreted carefully. This is particularly relevant in this study, as the reference genotype for qPCR-based expression comparisons was *C. sinensis*, while the hybrids were a cross of *C. sinensis* and *C. reticulata*. In other words, while expression of some genes was correlated with disease and pathogen infection, we were not able to fully demonstrate the causal relationship of resistance. Furthermore, variability is expected as to how hybrids will respond to various environmental challenges encountered in field conditions. Future work to address this question will require comparisons between uninfected and infected

hybrid lines, which should more conclusively link the *X. fastidiosa* resistance phenotype observed here to gene expression patterns.

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