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Molecular biology

Characterization of *Citrus sinensis* transcription factors closely associated with the non-host response to *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

Plants, when exposed to certain pathogens, may display a form of genotype-independent resistance, known as non-host response. In this study, the response of *Citrus sinensis* (sweet orange) leaves to *Xanthomonas campestris* pv. *vesicatoria* (Xcv), a pepper and tomato pathogenic bacterium, was analyzed through biochemical assays and cDNA microarray hybridization and compared with Asiatic citrus canker infection caused by *Xanthomonas citri* subsp. *citri*. Citrus leaves exposed to the non-host bacterium Xcv showed hypersensitive response (HR) symptoms (cell death), a defense mechanism common in plants but poorly understood in citrus. The HR response was accompanied by differentially expressed genes that are associated with biotic stress and cell death. Moreover, 58 transcription factors (TFs) were differentially regulated by Xcv in citrus leaves, including 26 TFs from the stress-associated families AP2-EREBP, bZip, Myb and WRKY. Remarkably, *in silico* analysis of the distribution of expressed sequence tags revealed that 10 of the 58 TFs, belonging to C2C2-GATA, C2H2, CCAAT, HSF, NAC and WRKY gene families, were specifically over-represented in citrus stress cDNA libraries. This study identified candidate TF genes for the regulation of key steps during the citrus non-host HR. Furthermore, these TFs might be useful in future strategies of molecular breeding for citrus disease resistance.

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Introduction

Plants are constantly exposed to a variety of pathogenic microorganisms. The results of plant–pathogen interactions depend on the combination of interacting partners, the plant developmental stage and environmental signals (Ascencio-Ibañez et al., 2008). A plant species is a "host" for a particular pathogen if at least one cultivar is susceptible to it. This dynamic, which leads to illness, is called "compatible interaction". Phytopathogens exhibit narrow "host" specificity and are unable to infect "non-host" plants due to non-host defense, a response considered "incompatible". Plant non-host resistance exhibits constitutive and inducible features that are not well understood at the molecular level (Nürnberger and Lipka, 2005). Non-host defense is recognized as a hypersensitive response (HR) if it presents visible symptoms. These symptoms result in localized cell death (LCD), with necrotic lesions that confine the pathogen at the infection site (Oh et al., 2006; Mur et al., 2008; Daurelio et al., 2011). The initial HR events following pathogen recognition include structural modifications, cytoplasmic membrane disruption, production of reactive oxygen species (ROS), synthesis of pathogenesis-related proteins (PRs) and transcriptional reprogramming (Eulgem, 2005; Oh et al., 2006; Daurelio et al., 2009b). The type of plant response depends on quantitative transcriptome modifications (Tao et al., 2003). Transcription factors (TFs) therefore play a fundamental role, and several of them have been shown to regulate the expression of defense-related genes (Singh et al., 2002; Eulgem, 2005). Accordingly, microarray technology has helped unravel key processes in plant responses to pathogens (Eulgem, 2005). The comprehension of transcriptional modifications involved in plant non-host resistance represents both a great scientific achievement and a fundamental step toward identifying genes for plant disease control breeding programs (Singh et al., 2002; Daurelio et al., 2011).

Abbreviations: CFU, colony forming units; Ctr, negative control; DAB, 3-3diaminobenzidine; EST, expressed sequence tag; HR, hypersensitive response; JA, jasmonic acid; LCD, localized cell death; pi, post infiltration; PR, pathogenesis-related protein; ROS, reactive oxygen species; SA, salicylic acid; TF, transcription factor; Xcv, *Xanthomonas campestris* pv. *vesicatoria*; Xcc, *Xanthomonas citri* subsp. *citri*.

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Although plant defensive mechanisms involve general pathways, they differ based on important traits between genera (van Loon et al., 2006). Moreover, while numerous studies have analyzed molecular defense mechanisms in herbaceous model plants such as *Arabidopsis*, tobacco, soybean and rice, little is known about them in tree crops of economic importance (Gomi et al., 2003). Citrus species are the most economically important fruit crop in the world due to their high productivity and the nutrient content of their fruits, yet they suffer devastating losses caused by pathogens (Talon and Gmitter, 2008). The transcriptional response of lemon (*C. limon*) leaves against non-pathogenic strains of *Alternaria alternata* (a citrus pathogenic fungus) using subtractive hybridization detected a reduced number of pathways involved in this interaction (Gomi et al., 2003). Recent advances in functional genomics and molecular biology, including the analysis of large expressed sequence tag (EST) collections in public databases (Forment et al., 2005; Campos et al., 2007; Guidetti-Gonzalez et al., 2007) and the design of different microarrays platforms (Fujii et al., 2007; Cernadas et al., 2008; Martinez-Godoy et al., 2008), have provided valuable tools for citrus–pathogen interaction research. Consequently, the citrus transcriptome was recently analyzed using microarrays during some compatible interactions (Mozoruk et al., 2006; Gandia et al., 2007; Cernadas et al., 2008; Kim et al., 2009). In addition, *in silico* analyses of PR- and HR-related ESTs have been carried out, but these were not confirmed experimentally (Campos et al., 2007; Guidetti-Gonzalez et al., 2007). To our knowledge, neither citrus non-host response characterization nor citrus non-host transcriptome analyses have been carried out to date. Although the citrus response to *Xanthomonas fuscans* pv. *aurantifolli* was defined as non-host, the symptoms manifested as an arrested canker instead of a typical HR (Cernadas et al., 2008). While the Key lime response to the citrus canker producing bacteria *Xanthomonas citri* subsp. *citri* (Xcc) strain A^W showed HR symptoms, the plant transcriptome was not analyzed during this host interaction (Rybak et al., 2009).

We studied the interaction between sweet orange leaves and the Gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* (Xcv), the agent responsible for spot disease in tomatoes and peppers (Thieme et al., 2005). The interaction was previously considered to be an HR (Daurelio et al., 2009a); this was confirmed in our study. In addition, we performed a microarray transcriptome analysis comparing the citrus–Xcv interaction against the compatible citrus–Xcc interaction to detect genes with modified expression specific to the citrus non-host response. Biotic stress, cell death and TF genes differentially expressed in the citrus response to Xcv were described. This study represents an initial effort to elucidate the mechanisms underlying the citrus non-host response to pathogens.

Materials and methods

Plant material, bacterial strains and inoculation procedure

Citrus sinensis L. Osbeck cv. Valencia Late plants generously provided by Catalina Anderson and Gastón Alanis (INTA Concordia, Argentina) were grown in a greenhouse at 25/18 °C (day/night) with a 14 h photoperiod (150 $\mu E\, m^{-2}\, s^{-1}$) and controlled relative humidity. Young, fully expanded leaves were used in all assays.

X. citri subsp. *citri* (Hasse) and *X. campestris* pv. *vesicatoria* (Doidge) strains were kindly provided by Blanca I. Canteros (INTA Bella Vista, Argentina). Strains were grown at 28 °C in SB medium (Daurelio et al., 2011) supplemented with 25 μg/mL ampicillin for Xcc. Bacterial overnight cultures were diluted to the indicated colony forming units (CFU)/mL in 10 mM $MgCl_2$ and pressure infiltrated into the abaxial side of the leaves using a syringe without a needle (Daurelio et al., 2009b). A solution of 10 mM $MgCl_2$ was used as a negative control (Ctr).

Infectivity study, histological analysis, ion leakage and growth curves

In the infectivity assays, phenotypes were discerned visually. To perform histological analysis, an inoculated region of approximately 1 cm^2 was excised at 48 h post infiltration (pi) and fixed in FAA solution (10% formaldehyde, 5% glacial acetic acid, 50% ethanol) for 24 h. After fixation, samples were dehydrated and embedded in paraffin (D'Ambrogio de Argüeso, 1986). Cross-sections were placed on glass slides, and paraffin was removed with xylene. Samples were stained with hematoxylin-eosin (Johansen, 1940) for observation by light microscopy.

Inoculated leaves were cut 2 hpi. Petioles were submerged in 1 mg/mL 3-3 diaminobenzidine (DAB), pH 3.8, for 18 h in darkness to detect hydrogen peroxide (H_2O_2) production (Daurelio et al., 2009b). Leaves were subsequently bleached and stored in 96% ethanol at room temperature.

Ion leakage measurements were performed to detect cytoplasmic membrane disruption. Leaf discs with a diameter of 1.3 cm were taken at 0, 4, 8 and 24 hpi. Leaf discs were rinsed for 10 min in 4 mL distilled water and incubated for 3 h in 1 mL fresh distilled water. Electrical conductivity was referred to as 100% ion content in boiled leaf discs (120 °C, 20 min) (Daurelio et al., 2009b). As a membrane disruption control, 1 mM salicylic acid (SA) was included. Assays were run in triplicate in independent leaves. A three-factor (treatment, time pi and leaf) mixed model ANOVA and a Tukey's multiple comparison test were used for statistical analyses. Residual analyses and validation with logarithmic data transformation were performed.

In planta growth assays were performed by grinding 1 cm^2 infiltrated leaf discs in 100 μL distilled water. These solutions were serially diluted and plated onto SB agar. Colonies were counted after 48 h of incubation at 28 °C and expressed as log CFU/cm^2 leaf tissue.

RNA isolation, sample labeling and microarray hybridization

Regions inoculated with Xcv, Xcc (10^7 CFU/mL) or Ctr were collected at 8 hpi. Tissue was harvested in liquid nitrogen and stored at −80 °C until RNA isolation. Total RNA was extracted using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol. RNA quality was tested using the OD_{260}/OD_{280} ratio and agarose gel electrophoresis (Sambrook et al., 1989). First-strand cDNA was generated from 1 μg total RNA. Double-stranded cDNA synthesis, *in vitro* transcription and amplification, labeling of amplified RNA, microarray hybridization and slide washes were performed as described by Cercós et al. (2006). RNA was extracted from three biological replicates, independently processed, labeled and hybridized to different microarrays. Individual RNA samples were used to synthesize Cy5-labeled antisense cRNA. These samples were co-hybridized with Cy3-labeled antisense cRNA that was synthesized from a reference sample containing a mixture of equal amounts of amplified RNA from all experimental samples (Agusti et al., 2008). Samples were labeled with Cy5 and the reference with Cy3 to avoid dye artifacts. A citrus cDNA microarray containing 21,081 putative unigenes was utilized (Martinez-Godoy et al., 2008).

Data acquisition and analysis

Hybridized arrays were scanned using a Scanarray Gx scanner (PerkinElmer) with Scanarray Express software following the manufacturer's instructions. An appropriate photomultiplier gain ratio for the two channels and a percentage of 1% of the saturated spots were obtained. GenePix 4.1 (Axon Instruments) was used to transform intensity measurements into numeric data. Data from spots flagged as "not found" or "bad" during scanning and spots with a signal to background ratio lower than 2 were discarded. Because

the comparisons performed revealed a low percentage of probes showing significant differences in expression, robust normalization was carried out using the Lowess method. Probes showing significant differential gene expression between samples were identified using the Linear Models in Microarrays (LIMMA) package (Smith, 2004) from the Bioconductor software library (Gentleman et al., 2004). Gene expression differences were considered to be significant when *p*-values (considering correction for false discovery rate) were lower than 0.01. The *M* value ($\log_2$ of expression ratio between treatments) cutoff was ±1 and was $M = \log_2$[Xcv/Ctr] for Xcv/Ctr, $M = \log_2$[Xcc/Ctr] for Xcc/Ctr and $M = \log_2$[Xcv/Xcc] for Xcv/Xcc comparisons. Venn diagrams were constructed using the Venny tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Functional classification of the selected EST subgroups was carried out over their *Arabidopsis thaliana* orthologs through MIPS (Munich Information Center for Protein Sequences, http://www.mips.gsf.de) and MapMan software (Thimm et al., 2004).

Full-length sequences of selected unigenes were made by sequential assembly of homolog ESTs. ESTs were retrieved using the Basic Local Alignment Search Tool BLASTN (Altschul et al., 1997), considering a query coverage of ≥10% and a max identity of ≥95%, from the National Center for Biotechnology Information EST database (http://www.ncbi.nlm.nih.gov/dbEST). Sequences were assembled using the CAP3 program (Huang and Madan, 1999). Protein sequences and domains were determined using ORF finder (Stothard, 2000) and Pfam (Finn et al., 2006) software, respectively.

Genes that were preferentially expressed in libraries associated with stress were identified using statistically based *in silico* analysis. A total of 514,845 citrus ESTs were categorized as stress or non-stress based on conditions used for library construction (see Supplementary Table S1). Paired comparisons were conducted using the Chi-squared test with IDEG6 (Romualdi et al., 2003). The numbers of stress/non-stress ESTs corresponding to each gene were used as the input parameters, in addition to the total number of ESTs grouped into each category (Supplementary Table S1). The differences in gene expression under stress conditions were considered statistically significant according to the Bonferroni correction.

Phylogenetic trees (5000 bootstrap) based on the neighbor-joining method (Saitou and Nei, 1987) were generated using MEGA4 (Tamura et al., 2007). The representative domains for citrus and *Arabidopsis* alleles used as input sequences were previously detected by in-batch Pfam analysis and extracted using a Daurelio–Kurth developed Perl script (www.perl.org).

Real-time RT-PCR

Gene expression was confirmed by real-time RT-PCR analysis. Primers were designed using Primer3 v.0.4.0 software (Rozen and Skaletsky, 2000). Analyzed ESTs, primers and product lengths are indicated in Supplementary Fig. S1. One microgram of total RNA from the same samples used in the microarray experiments was used for cDNA synthesis with the M-MuLV Retro Transcriptase enzyme (Promega, USA) and $d(T)_{22}$ oligonucleotide, following the manufacturer's instructions. PCR products using genomic DNA or cDNA templates for the actin housekeeping gene were sized differently, allowing for the detection of genomic DNA contamination. PCR reactions without the reverse transcription step did not yield products. Real-time PCR, specificity analysis using melting curves and data normalization to actin were performed as described in Daurelio et al. (2011).

Results

Characterization of the orange leaf–Xcv interaction

The response of *Citrus sinensis* leaves to Xcv treatment was compared with the response to Xcc treatment as the disease reference. Orange leaves showed necrosis in infectivity assays using 10^6, 10^7 and 10^8 CFU/mL Xcv inoculums between 48 and 72 hpi. Some chlorosis was observed when leaves were infiltrated with Xcv 10^5 CFU/mL, Xcc or Ctr (Fig. 1A). The initial symptoms of water soaking were observed in the abaxial region of leaves inoculated with Xcc (Fig. 1A).

The non-host response phenotype was confirmed at the cellular level by histological staining at 48 hpi. In comparison with Ctr, no

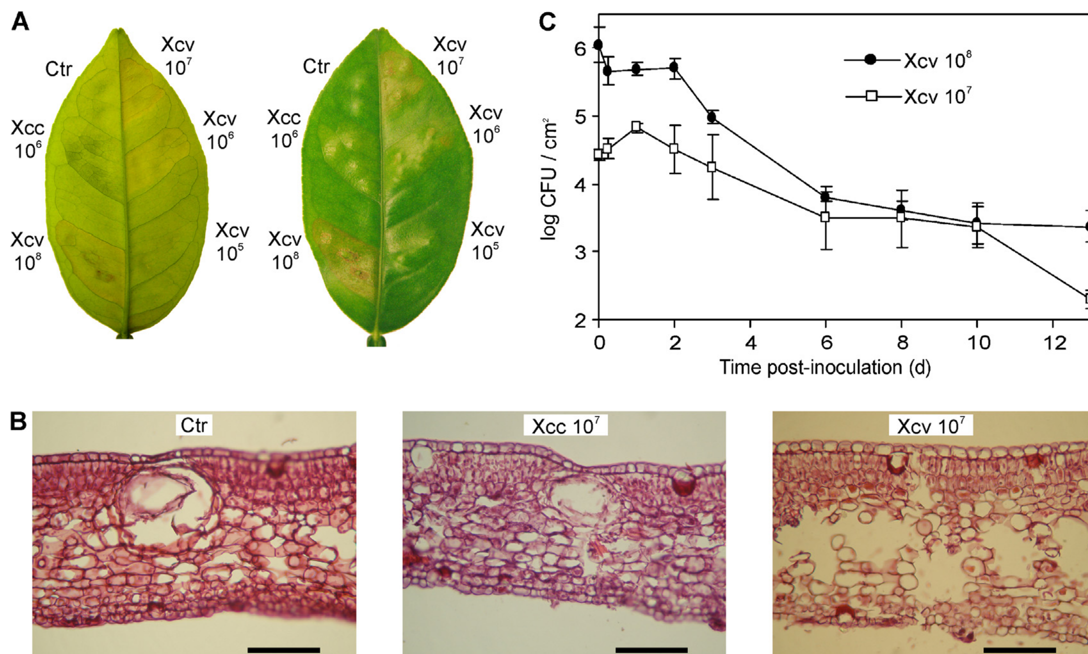

Fig. 1. Characterization of the citrus response to Xcv. (A) Inoculated leaves showing phenotypic responses on the abaxial (left) and adaxial (right) sides are observed at 72 hpi. (B) Histological analysis by hematoxylin-eosin staining showing leaf structure infiltrated with Ctr, Xcc and Xcv at 48 hpi (Bar = 50 μm). (C) Growth curves of Xcv in orange leaves. Values are averages of CFU/cm^2 recovered. Bars represent SD ($n = 3$). Inoculated bacteria dilutions are shown as CFU/mL.

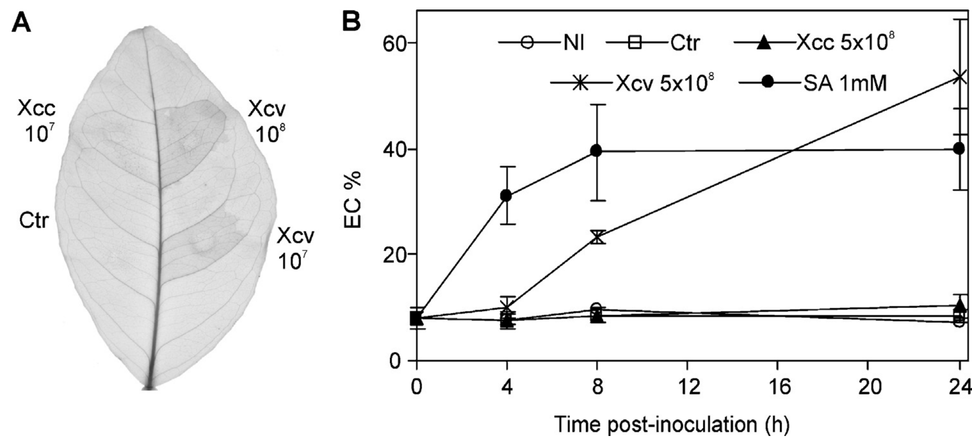

Fig. 2. Characterization of the citrus response to Xcv. (A) DAB staining at 2 hpi showing H_2O_2 localization in leaf tissue treated with Ctr, Xcc and Xcv. (B) Percentages of electrolytic conductivity for the treatments and non-infiltrated regions (NI) exhibited. Values are averages. Bars represent SD ($n = 3$). Inoculated bacteria dilutions are shown as CFU/mL.

microscopic changes were observed in the Xcc interaction. Xcv-inoculated leaves presented great areas of mesophyll cell lysis and complete structure disorganization (Fig. 1B).

During the study of Xcv behavior *in planta*, the number of recovered bacteria remained constant until 2 dpi. At 6 dpi, the bacterial population had diminished by two orders of magnitude for the largest initial inoculum and by one order for the smallest initial inoculum. In both cases, bacterial populations decreased slowly until 10 dpi (Fig. 1C).

Dark staining due to DAB oxidation by peroxidases in the presence of H_2O_2 was observed in Xcv inoculated leaves at 4 hpi. Xcc and Ctr treatments showed no such reaction (Fig. 2A). In ion leakage assays, no differences were observed between Ctr, Xcc and non-infiltrated regions. However, significant ion leakage increases were observed for Xcv at 8 and 24 hpi (Fig. 2B, $p < 0.001$).

Identification of differentially expressed genes during the citrus response to Xcv

Gene expression profiling was carried out on bacterial-infiltrated *Citrus sinensis* leaf tissue at 8 hpi using cDNA microarrays. Of the 21,081 putative unigenes in the citrus cDNA microarray, 2439 (11.5%) ESTs showed altered expression in both bacterial treatments, 1022 (42%) ESTs were induced, and 1417 (58%) were repressed (Fig. 3). Most of these genes were differentially regulated in the Xcv treatment compared with the Ctr (930 ESTs induced, 1309 ESTs repressed) or in the Xcv treatment compared with the Xcc treatment (732 ESTs induced, 813 ESTs repressed). Fewer differentially regulated ESTs were observed in the Xcc treatment compared to Ctr (64 ESTs induced, 71 ESTs repressed, Fig. 3).

Because the aim of this study was to detect genes specifically involved in the citrus non-host response, we evaluated those genes that were differentially expressed in Xcv when compared to Xcc and Ctr treatments (Fig. 3). Considering both subgroups, 654 probes were induced and 733 were repressed. Of these, 22 induced probes and 5 repressed probes were also differentially expressed in Xcc treatment compared to Ctr (Fig. 3). The respective *Arabidopsis* orthologs were assigned and functionally grouped (data not shown). Genes related to biotic stress and cell death and TF genes were analyzed with regard to the non-host response process.

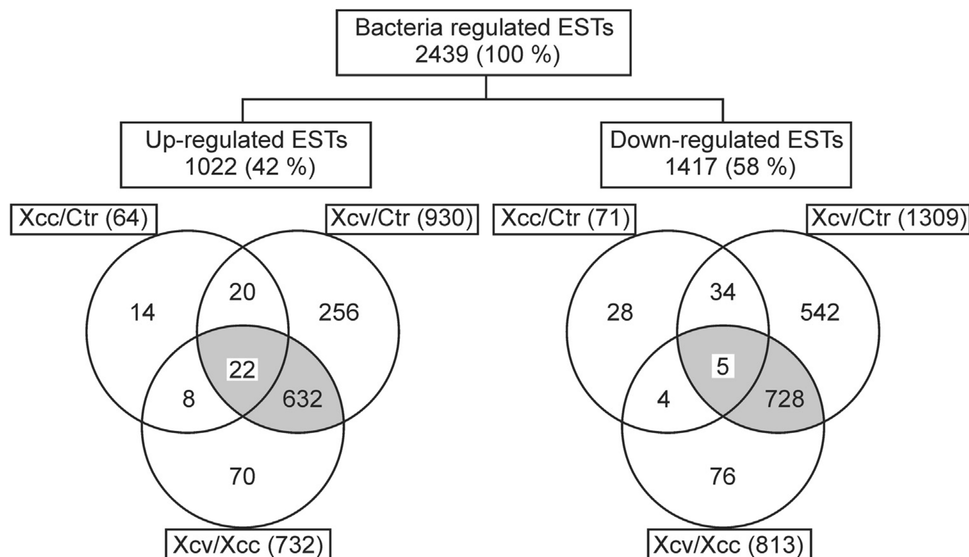

Fig. 3. Venn diagrams showing the number of orange ESTs differentially regulated at 8 hpi in leaf tissue during response to Xcv compared to Xcc treatment. The total numbers of ESTs are shown in boxes. Grey zones represent ESTs differentially expressed between Xcv/Xcc and Xcv/Ctr, or between Xcv/Xcc, Xcv/Ctr and Xcc/Ctr (white boxed numbers). Data are based on microarray analyses.

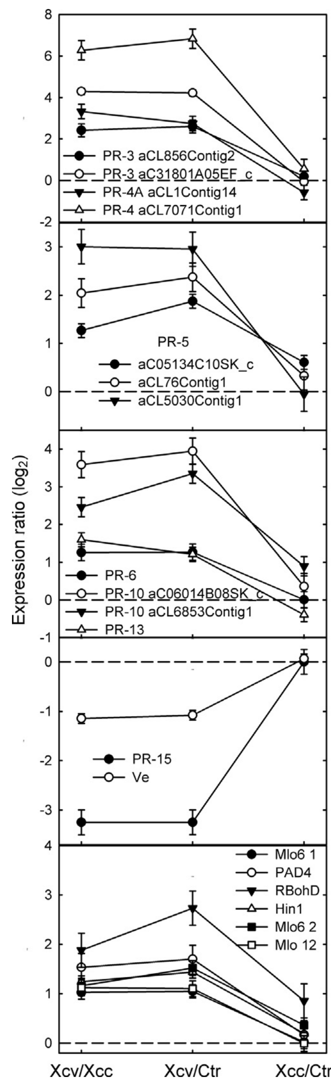

Fig. 4. Citrus ESTs that are differentially regulated during the non-host response to Xcv related to biotic stress. The $\log_2$ of expression ratio between treatments (*M*) are shown. Data are averages of three repetitions. Bars represent SE.

Biotic stress-related genes

Nineteen probes corresponding to 18 biotic stress-related genes were found to be differentially expressed (Fig. 4, Supplementary Table S2). Twelve of these genes showed homology to PRs as follows: two PR-3 and two PR-4 endochitinases that protect plants against fungi, nematodes and insects; three thaumatin-like PR-5 proteins with activity against oomycetes; one PR-6 proteinase inhibitor targeting nematodes and insects; two PR-10 enzymes with ribonuclease activity against viruses; one PR-13 or thionin-like protein with broad antibacterial and antifungal activity; and one PR-15 or germin-like protein (van Loon et al., 2006). The other six genes had the following homologies: two Mlo receptors (Mla powdery-mildew-resistant), a Ve-like (Verticillium wilt disease) leucine-rich repeat receptor protein, a lipase containing a domain similar to PAD4 (Phytoalexin Deficient 4), the NADPH oxidase RBohD and the inducible protein Hin-1 (hairpin type 1). All genes were induced in the Xcv treatment, except the germin-like protein (PR-15) and the homolog of the Ve-like receptor (Fig. 4).

PR-15 and Mlo6 were significantly under- and over-represented, respectively, under stress conditions. These results were consistent with the expression pattern observed in the orange leaf–Xcv interaction (Supplementary Table S2).

Microarray expression for one PR-4, RBohD and Hin-1 was confirmed by real-time RT-PCR analysis (Supplementary Fig. S1). Moreover, Mlo6 was represented by two different probes with similar expression ratios (Supplementary Table S2).

Genes involved in LCD

The response of orange leaves to Xcv inoculation showed HR features, suggesting the participation of LCD-related genes. Changes in gene expression were observed for five probes similar to cell death-associated genes; three probes were down-regulated and two probes were up-regulated (Table 1, Supplementary Table S3). The repressed genes showed homology to two cyclic nucleotide-gated ion channels called "defense-no-death" (Genger et al., 2008) and to a red chlorophyll catabolite reductase called "accelerated cell death 2" that catalyzes one step in the breakdown of the porphyrin component of chlorophyll (Yao and Greenberg, 2006). The induced genes showed similarity to "constitutively activated cell death 1" (Tsutsui et al., 2006) and to a cysteine protease cystatin-like inhibitor (Bozso et al., 2009). None of the five genes were predominantly expressed in libraries obtained under stress conditions (Supplementary Table S3).

Alleles homologous to TFs

The citrus microarray contained 899 probes corresponding to 590 *Arabidopsis* TFs. Of these, 58 probes analogous to 54 *Arabidopsis* TFs were differentially expressed in orange leaves in response to Xcv treatment (Supplementary Table S4). A group of 10 putative TFs with significant over-representation in citrus stress libraries included one C2C2-GATA, two C2H2, two CCAAT, one HSF, two NAC (No apical meristem-ATAF-cup-shaped cotyledon) and two WRKY

Table 1
Citrus ESTs that are differentially regulated during the non-host response to Xcv related to LCD. The $\log_2$ of expression ratio between treatments (*M*) are shown. The *M* contrast cutoff values of ±1 are shown in bold. Data are averages of three repetitions.

ID	Putative gene identification [*Species*] (Accession No.)	*E*-value	Putative Ath ortholog	*M* ± SE		
				Xcv/Xcc	Xcv/Ctr	Xcc/Ctr
C02006G06	Cyclic nucleotide-gated ion channel, putative [*Ricinus communis*] (XP_002532894)	0E+00	AT5G15410	**−1.39** ± 0.22	**−2.26** ± 0.22	−0.87 ± 0.22
C02011G07	Cyclic nucleotide-gated ion channel, putative [*Ricinus communis*] (XP_002525760)	0E+00	AT5G54250	**−1.15** ± 0.23	**−0.80** ± 0.23	0.36 ± 0.23
C31204D05	Red chlorophyll catabolite reductase ACD2, chloroplast precursor, putative [*Ricinus communis*] (XP_002523576)	4E−104	AT4G37000	**−0.99** ± 0.17	**−1.12** ± 0.17	−0.13 ± 0.17
C31401A10	Unnamed protein product, putative CAD1 [*Vitis vinifera*] (CBI18046)	0E+00	AT1G29690	**1.16** ± 0.17	**0.94** ± 0.17	−0.21 ± 0.17
KN0AAI1AA02	Predicted protein, putative cysteine protease inhibitor [*Populus trichocarpa*] (XP_002336103)	2E−16	AT3G12490	**1.21** ± 0.27	**1.30** ± 0.27	0.10 ± 0.27

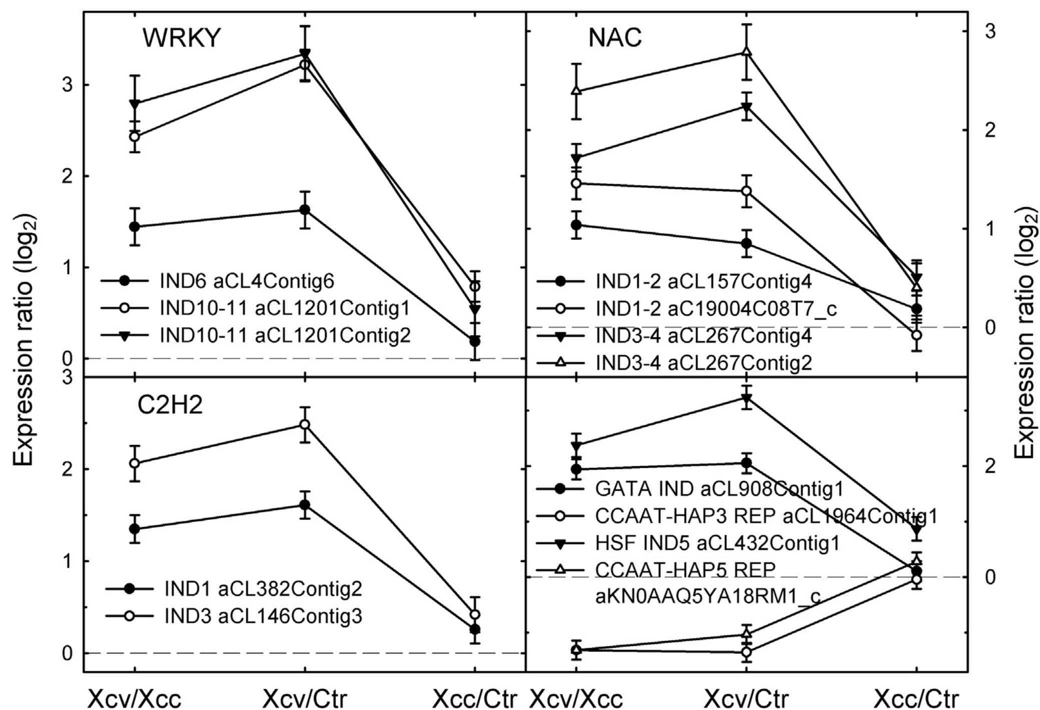

Fig. 5. Citrus ESTs that are differentially regulated during the non-host response to Xcv and correspond to TFs with significant representation in stress libraries. The $\log_2$ of expression ratio between treatments (M) are shown. Data are averages of three repetitions. Bars represent SE.

genes (Fig. 5). All of these putative TFs were induced in response to Xcv treatment, except both CCAAT genes (Fig. 5, Supplementary Table S4). Phylogenetic clustering of citrus and *Arabidopsis* TF domains showed that C2C2-GATA clustered with GATA-5-6-7 inside subfamily I, both C2H2 genes clustered with ZAT6 and STZ, CCAAT-HAP3 clustered with NF-YB3 within the NF-YB subfamily, CCAAT-HAP5 clustered with NF-YC11 inside the NF-YC subfamily, HSF clustered with HSFB1 of Group B, NAC genes clustered with ANAC047 in the NAP subfamily and with ATAF1-2 in the ATAF1 subfamily, and the WRKY genes grouped with AtWRKY11-17 inside subfamily II-d and with AtWRKY42-47 inside subfamily II-b (Supplementary Fig. S2).

Twenty six additional TFs that belong to families related to defense responses (Singh et al., 2002) showed differential expression in the citrus non-host response as follows: ten WRKY genes (eight induced, one repressed and one similarly induced in Xcv and Xcc treatments), five genes similar to the AP2-EREBP Ethylene Response Factor subgroup (one repressed, four induced), five Myb genes (four repressed, one induced) and four bZip genes (one repressed, three induced) (Supplementary Table S4).

The following genes with altered expression in microarray hybridization analyses were confirmed by real-time RT-PCR: one C2C2-GATA gene, one HSF gene, one Myb gene and two WRKY genes (Supplementary Fig. S1). In addition, six genes were represented by two different probes with similar expression ratios (Supplementary Table S4).

Discussion

Non-host response to Xcv in citrus shows HR-like symptoms

The non-host response against potential pathogens is the broadest and most important mechanism of plant defense, but it has mainly been studied in model plants. Citrus is the most important fruit crop in the world; however, the citrus non-host response and the associated transcriptome have not yet been studied. In a previous study, we observed that a response similar to the HR was induced in orange leaves by Xcv (Daurelio et al., 2009a). In this work, we studied the orange response to Xcv inoculations and the resulting transcriptome modifications. Because Xcc belongs to the same genus as Xcv, Xcc was used as the disease reference.

The orange leaves showed HR after Xcv infection. HR is a fast macroscopic collapse of infected tissue due to LCD (Mur et al., 2008). This symptom was directly observed at high and intermediate concentrations of bacterial inoculums but not at the lower concentration, suggesting that the intensity of the response depends on the initial concentration of bacteria. This phenotype was different than the canker symptoms seen in Xcc infection. The chlorosis observed at the lowest Xcv concentration and in Xcc inoculations can be considered to be non-specific or generated by the inoculation method because this phenotype also appeared in Ctr. The onset of HR-like and water soaking symptoms is fast, but this could be due to the young age of the leaves used for inoculation in these experiments. We have previously observed that symptoms appear earlier and more consistent in younger leaves compared to older leaves. The lysis of mesophyll cells, considered a typical symptom of the HR response (Danon et al., 2000), confirmed the phenotype observed.

The decrease in Xcv populations observed *in planta* can be considered typical in a non-host response (Daurelio et al., 2009b). This decrease was proportional to the initial bacterial concentration, suggesting that the activation of the defense response was more aggressive when greater numbers of bacteria are inoculated. Because the bacterial inoculums were infiltrated into mesophyll tissue, this response occurred independently of passive and preformed barriers. The determination of ion leakage and H_2O_2 production, indicators of plant HR symptoms (Daurelio et al., 2009b; Rybak et al., 2009), allowed us to confirm that the citrus response to Xcv treatment involved an active mechanism. This mechanism was considered to be a non-host response mediated by HR. Ion leakage values observed during Xcv treatment were comparable to those generated by SA used as control of cell death (Vlot et al., 2009). As seen in the grapefruit response to Xcc A^{w}, citrus phenotypic symptoms were slower than those observed for

other plant–pathogen interactions (Rybak et al., 2009). This finding suggests the participation of a mechanism that slows the negative effects of cell death.

Identification of genes that are expressed differentially during non-host response in citrus

One of the greatest challenges in plant science is the identification of plant transcriptome modifications responsible for defense or disease patterns. To find genes that trigger the citrus non-host response, transcriptome analysis was performed at 8 hpi. We observed that oxidative burst and membrane cell disruption (early symptoms) were occurring at this time, while necrosis and cell lysis symptoms manifested later. The comparison of Xcv and Xcc treatments allowed us to detect genes specifically involved in the citrus defense response. The higher number of differentially expressed genes in response to Xcv treatment was consistent with the quantitative nature of plant responses to pathogens (Tao et al., 2003). The number of genes that were up-regulated and down-regulated were similar, indicating an equivalent requirement of repression and induction during the defense response.

The transcription profiles determined by microarray analysis were confirmed using real-time RT-PCR. We attributed differences in absolute values to the dissimilar sensitivity of the two techniques. The validation of the microarray hybridization analysis was also supported for those genes with two probes that present similar expression values.

Genes related to biotic stress validate that the citrus response to Xcv is a non-host HR

The characterization of the citrus response to the Xcv treatment was confirmed by the differential expression of several genes related to the defense process. A homolog to Hin-1 was up-regulated; Hin-1 is a cell death-related gene that is typically induced in the non-host HR (Oh et al., 2006). In addition, a set of PRs associated with the non-host response (van Loon et al., 2006) were identified. The induced PRs were homologous to proteins that have defined roles in the protection against pathogens (van Loon et al., 2006). The *Arabidopsis* ortholog of citrus PR-15, which belongs to a receptor family involved in the stress response (Membre et al., 2000), is also repressed during biotic stress. This finding suggests that the protein participates in the citrus non-host response. Although an *in silico* analysis has identified PRs in citrus libraries (Campos et al., 2007), this work constitutes the first report describing their participation in the non-host response in citrus.

The *Arabidopsis* Mlo6 and Mlo12 genes are induced in the early response to flagellin (Navarro et al., 2004), while Mlo12 is also induced during non-host HR (Tao et al., 2003). Similarly, orthologous genes in citrus were induced. Mlo6 was found to be over-represented in citrus stress libraries, emphasizing its role in the defense response and possible involvement in pathogen recognition.

The homology and expression patterns observed for PAD4, RBohD and Ve2-like sequences identify them as the corresponding citrus alleles. The *Arabidopsis* PAD4 gene is involved in SA signaling during gene-mediated and basal plant disease resistance (Feys et al., 2001) and is induced in response to flagellin (Navarro et al., 2004). RBohD is involved in extracellular ROS production (Torres et al., 2002) and triggers death in cells damaged by fungal infection while inhibiting death in neighboring cells (Pogany et al., 2009). The Ve2 allele from *Solanum lycopersicum* does not participate in the response to pathogen attack and was repressed during an incompatible interaction (Fradin et al., 2009).

Genes associated with LCD could explain the citrus HR phenotype

The *Arabidopsis* LCD genes described in detail allowed us to investigate the citrus HR phenotype. The “defense-no-death” mutants carry out defense with a greatly reduced HR and have constitutively elevated SA levels (Genger et al., 2008). The “accelerated cell death 2” mutant shows the spontaneous spreading of cell death lesions and the constitutive activation of plant defense, including SA accumulation (Yao and Greenberg, 2006). The “constitutively activated cell death 1” mutant mimics HR lesions (induction of PR genes and increased SA concentration). This gene, induced by SA, regulates by negative feedback the SA-mediated defense pathway of cell death (Tsutsui et al., 2006). A cystatin-like gene is induced during defense responses to reduce the extent of LCD (Bozso et al., 2009).

In the citrus non-host response to Xcv treatment, the down-regulation of “defense-no-death” and “accelerated cell death 2” genes should lead to an increase in the SA concentration and trigger LCD. The increase in SA should induce “constitutively activated cell death 1”, which would negatively control SA and LCD levels. While down-regulation of “accelerated cell death 2″ leads to lesions of cell death, the diminishing of HR symptoms could be a consequence of “defense-no-death” down-regulation and cystatin-like up-regulation. This observation suggests that an improved defense mechanism diminishes deleterious effects.

LCD-related genes were not significantly expressed in citrus stress libraries, indicating their participation in other physiological processes.

TFs are candidates to trigger the non-host response in citrus

A group of differentially expressed TFs that are potentially involved in the onset of the citrus non-host response were detected. The most prominent candidates were those TFs that were significantly over-represented in citrus stress libraries and that showed specificity in the stress process. Those TFs related to plant defense may contribute in a non-specific way.

WRKY TFs are considered to be fundamental to plant defense (Eulgem, 2005). WRKY genes were the largest group of TFs differentially expressed during the citrus non-host response. Two WRKY TFs were induced and over-represented in citrus stress libraries. The aCL4Contig6 allele was similar to AtWRKY11-17, which are negative regulators of the basal response and inductors of genes related to jasmonic acid (JA) defense (Journot-Catalino et al., 2006). The aCL1201Contig1-2 allele was similar to the uncharacterized AtWRKY31 gene but grouped inside the subfamily IIb in which AtWRKY72 has been implicated in disease resistance (Bhattarai et al., 2010).

The aCL1506Contig1-aIC0AAA2BH06RM1_c allele was similar to AtWRKY33, a pathogen-induced TF required for resistance to necrotrophic fungal pathogens (Zheng et al., 2006). Three alleles (aCL775Contig1, aCL3000Contig1, and aIC0AAA56BD05RM1_c) were similar to the pathogen-induced gene AtWRKY53, which activates the defense response (Wang et al., 2006). These three genes were grouped in subfamily III with the pathogen-induced AtWRKY54-70, defined as negative regulators of SA synthesis (Wang et al., 2006). The aCL2994Contig1 allele was clustered in subfamily II-a with the pathogen-induced genes AtWRKY18-40-60, which have been shown to have redundant negative effects on SA and positive roles in JA-mediated defense (Xu et al., 2006). The aCL775Contig1 allele had similar expression levels in both bacterial treatments and could be involved in the basal response. This allele contains a WRKY domain similar to AtWRKY33, which regulates the antagonistic relationship between the defense responses to *Pseudomonas syringae* and necrotrophic fungal pathogens. The aC05807G12SK_c allele was similar to the AtWRKY75 and grouped

inside subfamily IIb, which has not been described yet. The only repressed WRKY allele (aCL1204Contig3) was similar to AtWRKY20 but grouped in subfamily I near AtWRKY58, a negative regulator of disease resistance (Wang et al., 2006). This finding coincides with the expression pattern observed.

The citrus Myb genes detected in the transcriptome were related to *Arabidopsis* orthologs that are not associated with the defense response. The Myb domain of the induced aC05146C04SK_c allele was homologous to NtMyb2, which positively regulates defense genes and is itself regulated during wounding and elicitor treatment (Sugimoto et al., 2003). NtMyb2 was transcriptionally activated by the GATA-type zinc finger protein AGP1 (Sugimoto et al., 2003). The AGP1 gene is homologous to the aCL908Contig1 TF, which was induced in response to Xcv treatment and was over-represented in citrus stress libraries. AGP1 induction by wounding was previously reported in tobacco (Sugimoto et al., 2003), but its participation in the non-host response to bacteria is reported for the first time here.

In addition to the WRKY and GATA TFs mentioned above, seven differentially expressed citrus TFs showed over-representation in citrus stress libraries. The induced C2H2 alleles (aCL382Contig2 and aCL146Contig3) were similar to the *Arabidopsis* STZ Zn finger protein that controls JA-regulated genes (Pauwels et al., 2008). The induced NAC allele aCL157Contig4-aC19004C08T7_c was similar to AtATAF1; this citrus allele grouped within the same subfamily but was nearest to the ATAF2, which is associated with regulation of the defense response in pepper (Oh et al., 2005). The induced NAC allele aCL267Contig2-4 has homology to a previously characterized citrus TF that was induced in different stresses (Fan et al., 2007) but not previously identified in the non-host defense response. The *Arabidopsis* alleles related to HSF (aCL432Contig1) and CCAAT (aCL1964Contig1 and aKN0AAQ5YA18RM1_c) were not previously studied during the non-host response. CCAAT genes were over-represented in stress libraries but were repressed during the citrus non-host response. This observation indicates that these genes have a possible ambiguous or specific function and need further characterization.

The induced AP2-EREBP allele aCL8155Contig1 was similar to AtERF1, which is induced during the *Arabidopsis* defense response to bacteria and has been proposed to be a connection between the ethylene and JA pathways in plant defense (Lorenzo et al., 2003). On the other hand, the induced AP2-EREBP aCL152Contig1 was similar to AtERF72. AtERF72 is induced in response to infection by the incompatible fungal pathogen *Alternaria brassicicola* (Okamuro et al., 1997). The aC08022H08SK_c TF showed sequence similarity to the bZIP gene At1HY5, a negative regulator of ethylene biosynthesis via the activation of AtERF11.

In conclusion, we have characterized the citrus non-host response to Xcv treatment and obtained evidence that the response mechanism is similar to HR. Transcriptional profiling supported this hypothesis and further explained the phenotype observed. The transcriptional survey and the *in silico* analysis of citrus EST distribution allowed us to identify several TFs that are potentially responsible for triggering the non-host response in citrus. These selected TFs could be used in molecular breeding to improve plant defense. We identified potential regulatory genes in the non-host response in citrus that are novel. These data make it possible to hypothesize about this mechanism in other woody crop species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2013.01.011.

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