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Novel insights into the *Citrus sinensis* nonhost response suggest photosynthesis decline, abiotic stress networks and secondary metabolism modifications

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Abstract. Plants are constantly exposed to stress factors. Biotic stress is produced by living organisms such as pathogens, whereas abiotic stress by unfavourable environmental conditions. In *Citrus* species, one of the most important fruit crops in the world, these stresses generate serious limitations in productivity. Through biochemical and transcriptomic assays, we had previously characterised the *Citrus sinensis* (L.) Osbeck nonhost response to *Xanthomonas campestris* pv. *vesicatoria* (Doidge), in contrast to Asiatic citrus canker infection caused by *Xanthomonas citri* subsp. *citri* (Hasse). A hypersensitive response (HR) including changes in the expression of several transcription factors was reported. Here, a new exhaustive analysis of the *Citrus sinensis* transcriptomes previously obtained was performed, allowing us to detect the over-representation of photosynthesis, abiotic stress and secondary metabolism processes during the nonhost HR. The broad downregulation of photosynthesis-related genes was correlated with an altered photosynthesis physiology. The high number of heat shock proteins and genes related to abiotic stress, including aquaporins, suggests that stresses crosstalk. Additionally, the secondary metabolism exhibited lignin and carotenoid biosynthesis modifications and expression changes in the cell rescue GSTs. In conclusion, novel features of the *Citrus* nonhost HR, an important part of the plants' defence against disease that has yet to be fully exploited in plant breeding programs, are presented.

Additional keywords: canker, hypersensitive response, pathogen, sweet orange, *Xanthomonas campestris* pv. *vesicatoria*, *Xanthomonas citri* subsp. *citri*.

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Introduction

Plants are sessile organisms exposed to a broad range of pathogens. During compatible interactions, virulent pathogens successfully invade and colonise the host tissue, leading to plant disease (Agrios 2005). Virulent microorganisms modulate the host tissues to avoid recognition and can delay the defence responses until they will not be effective anymore, with the aim of exploiting the host tissues as a source of energy (Senthil-Kumar and Mysore 2013). Despite this, plants possess a complex and effective set of defensive barriers to prevent the invasion and spread of the pathogen (Mysore and Ryu 2004). This process is initiated after recognition of pathogen-associated molecular patterns by cell surface receptors in the host cell, triggering the subsequent activation of regulatory proteins, protein kinases and transcription factors as part of the basal response (Chisholm *et al*. 2006; Jones and Dangl 2006). The incompatible interaction known as host resistance confers resistance to specific avirulent strains of a virulent pathogen, because it is recognised very early in the infection and the plant can effectively defend itself. On the other hand, the incompatible interaction called the nonhost response is the most widespread in plants and it is generally responsible for resistance against all races of potential pathogens (Senthil-Kumar and Mysore 2013). The hypersensitive response (HR) is a distinctive plant defence developed during incompatible interactions and it is characterised by tissue necrosis and a rapid and localised cell death at the site of infection (Senthil-Kumar and Mysore 2013). During HR, several defence responses

are activated such as reactive oxygen species (ROS) production, expression of proteins with antimicrobial properties, cell wall strengthening surrounding the infected region and activities involved in phytoalexin biosynthesis (Senthil-Kumar and Mysore 2013).

Pathogens have a big impact on the host plant's metabolism (Bolton 2009). During a susceptible interaction, the virulent pathogen is capable of withdrawing sugars from the host plant, increasing its consumption of C compounds. In this context, it was reported that several pathogens regulate the SWEET family transporters to induce the sugar efflux outside of the host cell and also induce sink genes such as cell wall invertases (Göhre *et al*. 2012). The induction of the resistance response requires energy and also a C source to synthesise defensive compounds. However, in contrast to the expected increase in photosynthesis during virulent or avirulent pathogen infections, a marked decrease in this process has been observed (Göhre *et al*. 2012). This phenomenon was shown by evaluation of photosynthetic parameters, proteomics and transcriptomics assays (Zou *et al*. 2005; Göhre *et al*. 2012). It has been suggested that the decrease in photosynthesis during infections could be an active process of the plant defence program to limit C source accessibility for the pathogen or as a consequence of a prioritisation of metabolic processes in favour of defence reactions (Bolton 2009). It has also been related to the chloroplast participation in ROS production during defence through photosynthetic electron transfer disruption and the consequent formation of highly reactive intermediates in light-exposed green tissues, as well as the release of chlorophyll, leading to the accumulation of phototoxic intermediates (Kangasjarvi *et al*. 2012).

An extensive overlap and crosstalk between the signalling pathways addressing biotic and abiotic stress responses has been proposed. Some reports showed that prior exposure to abiotic stress enhances disease resistance (Van Bockhaven *et al*. 2013), indicating that signal interactions can be both synergistic and antagonistic. The elucidation of the molecular mechanism that regulates this crosstalk is an important goal to be determined (Van Bockhaven *et al*. 2013). In addition, secondary metabolites, including terpenoid, phenylpropanoid and N-containing substances, are produced by different pathways, and they play essential roles in the plant's protection against pathogens (Makkar *et al*. 2007). Among the phenylpropanoids, the lignin pathway is activated during cell-wall reinforcement, which occurs by deposition of lignin and phenolic related compounds (Xu *et al*. 2011).

Citrus species are one of the most important fruit crops in the world and an important part of the yields is lost due to pathogen attack (Talon and Gmitter 2008). Knowledge of the defence strategies used by perennial tree plants like citrus during biotic stress is very important to prevent disease losses and it would also complement the studies performed in herbaceous model plants (Jones and Dangl 2006). Several genetic, genomic and proteomic tools have been quickly adopted in recent years by the citrus research community to address the major challenges of this fruit crop (Talon and Gmitter 2008). Transcriptomic approaches with microarrays have been used to study the molecular processes underlying the response of *Citrus* to biotic and abiotic stresses (Gimeno *et al*. 2009; Daurelio *et al*. 2013).

Previously, we studied the interaction of *Citrus sinensis* (L.) Osbeck (sweet orange) leaves with the Gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* (Doidge) (*Xcv*), described as a HR-like nonhost response (Daurelio *et al*. 2009). In a more recent work we employed biochemistry assays and transcriptomic analysis to characterise and evaluate the global expression profile of the *C. sinensis* response to *Xcv* in comparison with the citrus canker disease caused by *Xanthomonas citri* subsp. *citri* (Hasse) (*Xcc*). The *C. sinensis–Xcv* interaction was confirmed as a nonhost response mediated by HR, and several differentially expressed genes related to biotic stress, cell death and transcription factors were described (Daurelio *et al*. 2013). For that purpose, we used a cDNA microarray containing up to 20 000 probes (Martinez-Godoy *et al*. 2008), developed by the Spanish Citrus Functional Genomic Project (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/, accessed 29 April 2015).

In this survey, we carried out an analysis of the previously obtained transcriptomic data with the aim of identifying the novel processes involved in the *Citrus* nonhost response to bacteria. This study allowed us to detect photosynthesis, secondary metabolism and abiotic stress pathways as being over-represented categories during the nonhost response to *Xcv* in comparison to *Xcc* (Daurelio *et al*. 2013). The modifications suffered by these processes at the transcriptomic level were corroborated by means of molecular and physiological assays in plants. These results have remarkably increased the current list of genes and gene families related to the mechanisms underlying induced resistance in *Citrus*, results that can be extrapolated to other woody trees and plants in general.

Materials and methods

Plant, strains and inoculation procedures

The *Citrus sinensis* (L.) Osbeck cv. Valencia Late plants used in this work were kindly provided by Catalina Anderson and Gastón Alanis (Instituto Nacional de Tecnología Agropecuaria Concordia, Argentina). They were cultivated in a greenhouse at 25°C : 18°C (day : night) under controlled relative humidity with a 14-h photoperiod (150 $\mu E\,m^{-2}\,s^{-1}$). Young, entirely expanded leaves were used in the experiments.

Xanthomonas citri subsp. *citri* (Hasse) and *Xanthomonas campestris* pv. *vesicatoria* (Doidge) strains were generously supplied by Blanca I. Canteros (Instituto Nacional de Tecnología Agropecuaria Bella Vista, Argentina). Strains were grown in Silva-Buddenhagen (SB) medium (Daurelio *et al*. 2011) at 28°C, supplemented with 25 $\mu g\,mL^{-1}$ ampicillin for *Xcc*. The overnight cultures of bacteria were diluted to 10^7 colony forming units per mL in 10 mM $MgCl_2$ and were pressure infiltrated into the abaxial side of the leaves using a needleless syringe (Daurelio *et al*. 2011). A solution of 10 mM $MgCl_2$ was used as a control.

Microarray data acquisition and analysis

Microarray data for the *C. sinensis* response to *Xcv* in comparison to *Xcc* and the control were obtained from Daurelio *et al*. (2013).

In this work, differentially expressed genes between *Xcv* and *Xcc*, and between *Xcv* and the control were analysed in order to identify differentially regulated biological processes. This analysis was performed by two independent web-based platforms: the GeneTrail – advanced gene set enrichment analysis (Backes *et al*. 2007) and the agriGO–GO Analysis Toolkit and Database for Agricultural Community (Du *et al*. 2010). Because similar results were obtained with both tools, just those obtained via agriGO were presented. These methods allow the detection of over-represented and under-represented functional categories in a given gene set using *Arabidopsis thaliana* (L.) Heynh. genes, so the *A. thaliana* orthologues for *C. sinensis* alleles were obtained from the microarray platform (Martinez-Godoy *et al*. 2008). The results were also correlated with the classification made by MapMan software (Thimm *et al*. 2004).

Phylogenetic trees (5000 bootstraps) based on the neighbour–joining method were generated with the MEGA4 program as previously described (Daurelio *et al*. 2013). The representative domains for *C. sinensis* and *A. thaliana* alleles were used as input sequences; these had previously been detected by in-batch Pfam analysis and extracted using a custom developed Perl script (www.perl.org, accessed 29 April 2015).

Chlorophyll fluorescence and pigments determination

Chlorophyll fluorescence parameters (Baker 2008) were measured using a portable pulse amplitude modulation fluorometer (Qubit Systems Inc., Kingston, Ontario, Canada) connected to a notebook computer (Acer Aspire 7104WSMi, model number MS2195, Acer America Corporation, San Jose, CA, USA) with data acquisition software (Logger Pro3 Version, Vernier Software & Technology, Beaverton, OR, USA). The minimal fluorescence level (F_0) in the dark-adapted state was measured when only the light-emitting diode light was turned on. The output from the light-emitting diode light was insufficient to drive photosynthesis and did not disturb the dark-adapted state. The maximal fluorescence level in the dark-adapted state (F_m) and the maximal fluorescence level during illumination (F_m') were measured with a 0.8-s saturating pulse at 5000 µmol m^{-2} s^{-1}. F_m was measured after 30 min of dark adaptation. F_m' was measured with an actinic light source with a PPFD 100 µmol m^{-2} s^{-1}. The minimal fluorescence level during illumination (F_0') was calculated from measured values of F_0, F_m, F_m' and F_v (Baker 2008). The maximum quantum efficiency of PSII (F_v/F_m), the PSII maximum efficiency (F_v'/F_m') and the PSII operating efficiency ((F_m'/F_s) F_m'–1) were calculated as previously described (Baker 2008). Photochemical quenching (qP) was calculated as ($F_m' - F_s$) ($F_m' - F_0$)$^{-1}$ and permitted us to calculate the pressure of excitation as $1 - qP$. The nonphotochemical quenching (NPQ) was calculated as (F_v/F_m') – 1. The effective PSII quantum yields of regulated nonphotochemical energy dissipation (Y(NPQ)) and nonregulated energy dissipation (Y(NO)), which are complementary with the photochemical quantum yield (Y(II)), where $Y(II) + Y(NPQ) + Y(NO) = 1$, were calculated as previously reported (Baker 2008).

For pigment measurements, discs of 1 cm^2 were excised from *C. sinensis* leaves and placed into plastic tubes with 1 mL 100% (v/v) ethanol, sealed and incubated in the dark at 60°C for 48 h. Absorbance of the clear extract at 649 nm, 665 nm and 750 nm were recorded, and the concentrations of chl *a*, chl *b*, chl *a* + *b* and carotenoids were calculated as previously described (Lichtenthaler 1987).

Histological analysis

To perform histological analysis, an inoculated region of ~1 cm^2 was excised at 48 h after inoculation. Longitudinal sections 10 µm thick were cut with a MICROM HM500 cryostat (Global Medical Instrumentation, Ramsey, MI, USA). Several sections were stained in an aqueous solution of 0.01% acridine orange (w/w) at room temperature. After washing in water, the stained sections were mounted on glass slides and they were examined with a computer-controlled advanced microscope system (TE2000E2, Nikon, Tokyo, Japan) equipped with an optic system (DIC/Nomarski, Nikon) corrected to infinite, using 460 nm for excitation and 561 nm for emission.

On the other hand, lignin histochemistry was examined using Wiesner reagent (phloroglucinol-HCl, Phloroglucinol, Sigma-Aldrich, St Louis, MO, USA). Cross-sections of tissue leaves were incubated for 10 min in a phloroglucinol solution (2% (w/v) in 95% (v/v) ethanol), treated with 18% (v/v) HCl for 5 min and directly observed using a Leica fluorescence microscope (DM2500, Leica, Wetzlar, Germany).

Real-time reverse transcription–PCR

Gene expression obtained by microarray data was confirmed using real time reverse transcription–PCR assays. Primers were designed using Primer3 ver. 0.4.0 software (Rozen and Skaletsky 2000). Analysed alleles, primers and product lengths are shown in Table S4, available as Supplementary Material to this paper. 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, Madison, WI, USA) and d(T)$_{22}$ oligonucleotide, following the manufacturer's instructions. In addition, PCR products using genomic DNA or cDNA templates for the actin housekeeping gene had different sizes, allowing the detection of genomic DNA contamination. PCR reactions without the reverse transcription step did not yield products. Real-time PCR was performed with a Realplex Instrument (Eppendorf, Hamburg, Germany) equipped with Realplex Software ver. 4.0 (Eppendorf). Reactions were performed with 1 µL of cDNA template and a SYBR green-I reaction mixture containing 1 : 50 000 diluted SYBR green-I (Thermo Fisher Scientific, Waltham, MA, USA), 10 pmol of each primer, 0.5 U Platinum-Taq DNA polymerase (Invitrogen), 40 mmol deoxynucleotide triphosphates (dNTPs), 3.75 mM $MgCl_2$ and 1× Platinum-Taq buffer in a final volume of 20 µL under the following conditions: 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 59°C for 20 s and 72°C for 40 s. Fluorescent intensity data were acquired during the 72°C extension step. The specificity of the amplification reactions was assessed by melting curve analysis; the assays were run at 95°C for 15 s and 60°C for 15 s, followed by an increase in temperature from 60°C to 85°C (0.2°C s^{-1}) with continuous fluorescence recording. To perform the analysis of relative

expression, we used the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) normalising to the actin allele.

Statistical analysis

To identify differentially regulated biological processes, a singular enrichment analysis was used, with the hypergeometric statistical test and Hochberg FDR correction, using the *A. thaliana* orthologues of the differentially expressed *C. sinensis* genes and the *A. thaliana* genome as a background.

The chlorophyll fluorescence parameters and the chl *a* and carotenoid contents were statistically analysed using a three-factor (treatment, time after inoculation and leaves) mixed-model ANOVA and LSD multiple comparison tests along with residual analysis and validation. Infectivity response experiments were repeated at least three times to ensure the reproducibility and consistency of the results.

Results

Identification of differentially regulated biological processes

Analysis of the transcriptomic results obtained previously (Daurelio *et al*. 2013) allowed us to identify the biological processes that were differentially regulated in the *C. sinensis* nonhost response to *Xcv* in comparison to the disease pattern generated by *Xcc* at 8 h after infection. Orthologous *A. thaliana* genes were identified for 1210 of the 1387 differentially expressed genes, 1051 of which were successfully categorised in the agriGO program. Analysis of these genes revealed that the functional categories 'photosynthesis', 'response to stimulus', 'aromatic compound biosynthetic processes' and 'secondary metabolic processes' were over-represented during the *C. sinensis* nonhost response to *Xcv* (Table 1, Fig. S1). The most prominent group was 'photosynthesis' with 29 genes, 22 of which participate in light reactions. The category 'response to stimulus' showed 232 genes, 146 being classified as response to stress, including several heat shock proteins (HSPs) and genes that participate in response to abiotic stress. The category 'secondary metabolism', with 38 genes, covers an important group of enzymes, several of which are involved in phenylpropanoid pathways, mainly lignin biosynthesis and cell rescue, which are key components of the defence response.

Photosynthesis declines during the C. sinensis *nonhost response*

The photosynthesis-related genes were all downregulated during the *C. sinensis* nonhost response to *Xcv* including genes of PSI, such as subunits of the reaction centre and chlorophyll-binding proteins; genes of PSII, such as O-evolving enhancer proteins, subunits of the reaction centre, light-harvesting complex (LHC) subunits, chlorophyll binding proteins and FtsH proteases of subunit D; genes involved in electron transport, such as cytochrome B6-F complex subunits, ferrodoxin : $NADP^+$ oxidoreductase and plastocyanin; and subunits of the ATPase complex (Table S1). On the other hand, one cell wall invertase and three hexoses transporters were upregulated; these were not classified in photosynthesis category but were involved in the primary metabolism modification during the nonhost response (Table S1).

In order to determine whether this transcriptional downregulation was accompanied by an altered photosynthesis physiology, chlorophyll fluorescence parameters were measured at 1, 2 and 3 days after inoculation (DAI). The maximum quantum efficiency of PSII in the dark adapted state (F_v/F_m), the PSII operating efficiency (ϕ_{PSII}) and the PSII maximum operating efficiency ($\max\phi_{PSII}$) were significantly lower under the *Xcv* treatment ($P < 0.05$, Fig. 1*a*). No differences were observed for NPQ, although a higher value of pressure excitation was observed in *Xcv*-inoculated leaves ($P < 0.05$, Fig. 1*a*). These differences in the *Xcv* treatment were noted at 1, 2 and 3 DAI, whereas differences between *Xcc* and the control were observed at 2 or 3 DAI, probably as a consequence of canker disease symptoms (Kraiselburd *et al*. 2013) (Fig. 1*a*).

The excitation energy absorbed in PSII may follow three possible pathways. Chlorophyll fluorescence allows the measurement of this excitation energy flux into photochemical consumption or PSII operating efficiency ($\phi_{PSII} = Y(II)$) and to estimate the regulated heat dissipation process acting as protection ($\phi_{NPQ} = Y(NPQ)$) and the nonregulated heat dissipation process due to PSII inactivity (Y(NO)). The

Table 1. Over-representation analysis of differentially expressed gene families during the *Citrus sinensis* nonhost response to *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*)

A singular enrichment analysis, with the hypergeometric statistical test and the Hochberg false discovery rate (FDR) correction was performed, using the *Arabidopsis thaliana* orthologues of the differentially expressed genes and the *A. thaliana* genome as a background. The number of genes with modified expression during the response (input list), the number of genes represented in the microarray (reference), *P*-value for the respective analysis and FDR correction are indicated for each Gene Ontology category (GO term)

GO term	Description	Number in input list	Number in reference	*P*-value	FDR
GO:0050896	Response to stimulus	232	1521	2.1×10^{-8}	5.4×10^{-6}
GO:0042221	Response to chemical stimulus	138	786	2.8×10^{-8}	5.4×10^{-6}
GO:0006950	Response to stress	146	880	2.3×10^{-7}	3×10^{-5}
GO:0015979	Photosynthesis	29	85	6.2×10^{-7}	6×10^{-5}
GO:0019684	Photosynthesis, light reaction	19	46	6×10^{-6}	0.00047
GO:0019438	Aromatic compound biosynthetic process	28	107	6×10^{-5}	0.0039
GO:0019748	Secondary metabolic process	38	174	9.7×10^{-5}	0.0054
GO:0006091	Generation of precursor metabolites and energy	30	128	0.00018	0.0088

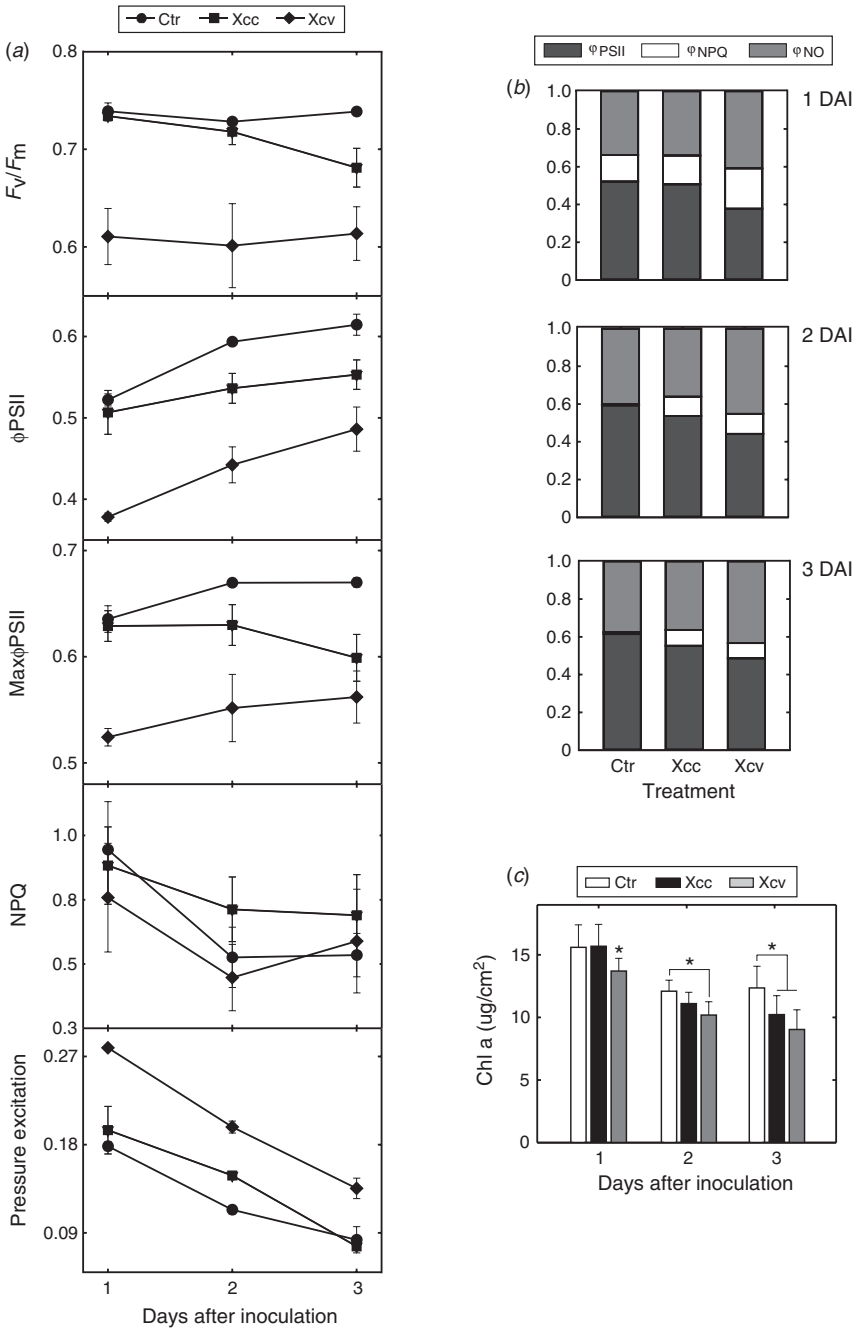

Fig. 1. Analysis of the photosynthetic process during the nonhost response in *Citrus sinensis*. (*a*) The chlorophyll fluorescence parameters analysed were: the maximum quantum efficiency of PSII in the dark adapted state (F_v/F_m), PSII operating efficiency (φPSII), PSII maximum operating efficiency (maxφPSII), nonphotochemical fluorescence quenching (NPQ) and pressure of excitation. Treatments are indicated at the top of the figure: Ctr, control; *Xcc*, *Xanthomonas citri* subsp. *citri*; *Xcv*, *Xanthomonas campestris* pv. *vesicatoria*. The results are the mean of three replicates and error bars represent the s.e. Mixed-model ANOVA and LSD multiple comparison tests were used for statistical analysis. (*b*) Flow of excitation energy in PSII: quantum yields of PSII photochemistry (φPSII), light-induced quenching processes (φNPQ) and nonlight-induced quenching processes (φNO) were determined in the treatments indicated at 1, 2 and 3 days after inoculation (DAI). (*c*) Chl a content measurement of bacteria-infiltrated *C. sinensis* leaves. Treatments are indicated at the top of the figure. The results are the mean of three replicates and error bars represent the s.e. Mixed-model ANOVA and LSD multiple comparison tests were used for statistical analysis. Asterisks indicate significant differences between *Xcv* versus *Xcc* and Ctr (1 DAI), *Xcv* versus Ctr (2 DAI), and *Xcv* and *Xcc* versus Ctr treatments (3 DAI) ($P < 0.05$).

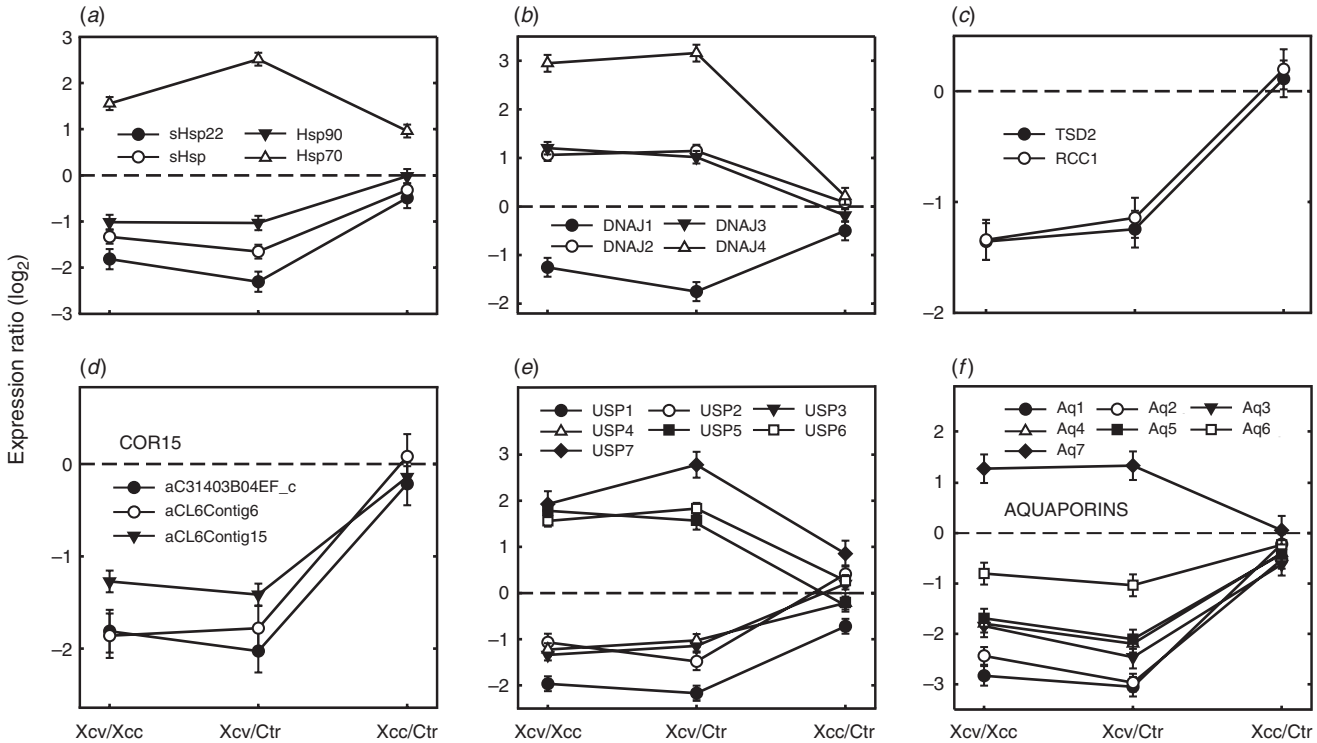

Fig. 2. *Citrus sinensis* 'response to stress' genes differentially regulated during the nonhost response to *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). The $\log_2$ of the expression ratio between treatments are shown. The results are the mean of three replicates and error bars represent the s.e. HSP, heat shock protein; sHSP, small HSP; USP, universal stress protein; *RCC1*, *regulator of chromosome condensation 1*; *TSD2*, *tumorous shoot development 2*; Aq, aquaporin; Ctrl, control; *Xcc*, *Xanthomonas citri* subsp. *citri*. (*a*) *sHSP22*, *sHSP*, *HSP90* and *HSP70*; (*b*) *DNAJ1*, *DNAJ2*, *DNAJ3* and *DNAJ4*; (*c*) *TSD2* and *RCC1*; (*d*) *aC31403B04EF_c*, *aCL6Contig6* and *aCL6Contig15*; (*e*) *USP1*, *USP2*, *USP3*, *USP4*, *USP5*, *USP6* and *USP7*; (*f*) *Aq1*, *Aq2*, *Aq3*, *Aq4*, *Aq5*, *Aq6* and *Aq7*. Contigs corresponding to the alleles observed are (see also Table S2): *aIC0AAA81CA10RM1_c* (*sHSP22*), *aCL4866Contig1* (*sHSP*), *aC31002F12EF_c* (*HSP90*), *aC34105F11EF_c* (*HSP70*); *aCL2805Contig2* (*DNAJ1*), *aCL7431Contig1* (*DNAJ2*), *aC32105E10EF_c* (*DNAJ3*), *aC06004B03SK_c* (*DNAJ4*), *aCL4197Contig1* (*TSD2*), *aC01010D10SK_c* (*RCC1*), *aKN0AAP7YE17FM1_c – aCL4481Contig1* (*USP1a–b*), *aC01018F10SK_c – aCL2470Contig1* (*USP2a–b*), *aCL5203Contig1* (*USP3*), *aKN0AAP5YG22FM1_c – aCL3202Contig1* (*USP4a–b*), *aCL102Contig2* (*Aq1*), *aCL1621Contig2* (*Aq2*), *aCL165Contig1* (*Aq3*), *aCL3500Contig1* (*Aq4*), *aCL58Contig7* (*Aq5*), *aCL3429Contig1* (*Aq6*), *aCL4824Contig1* (*Aq7*).

fraction of the PSII excitation energy leading to noncontrolled heat dissipation (Y(NO)) was bigger under the *Xcv* treatment in comparison to the control or the *Xcc* treatment at 1, 2 or 3 DAI (Fig. 1*b*). The increase in Y(NO) was paralleled by a decrease in ϕ_{PSII}, which indicates inhibition of photosynthesis (Fig. 1*b*); Y (NPQ) levels remained constant except for in the control, where it diminished at 2 and 3 DAI (Fig. 1*b*), possibly as consequence of the recovery of infiltration damage.

On the other hand, several *C. sinensis* genes related to chlorophyll metabolism presented modifications in their expression patterns. The negative regulator of biosynthesis 'fluorescent in blue light' (Flu) and the biosynthetic enzymes coproporphyrinogen-III oxidase, magnesium-protoporphyrin IX monomethyl ester cyclase (CHL27), magnesium-chelatase (CHLH), protoporphyrinogen oxidase (PPOX) and protochlorophyllide oxidoreductase (PORA) were repressed, whereas the catabolic enzyme chlorophyllase 1 (Chl1) was induced, promoting chlorophyll degradation, and the catabolic enzyme pheophorbide a oxygenase was repressed, reducing the degradation in the downstream steps (Table S1). In addition, two alleles that were similar to early light-induced proteins, specialised chlorophyll-binding proteins homologous to LHC, were also repressed (Table S1).

The expression patterns suggested chlorophyll diminution, so chlorophyll content (chl *a* and chl *b*) were quantified at 1, 2 and 3 DAI. The analysis of the pigments showed no difference between treatments for chl *b* (data not shown), whereas chl *a* levels decreased substantially after 1 DAI in leaves inoculated with *Xcv* ($P < 0.05$, Fig. 1*c*) and exhibited similar values to the control in leaves inoculated with *Xcc*. At 2 DAI, a significant decrease was observed for the *Xcv* treatment in comparison to the control ($P < 0.05$, Fig. 1*c*), although *Xcc* showed an intermediate value without differences from the control or *Xcv*. At 3 DAI, chl a levels were similar for the *Xcv* and *Xcc* treatments, lower than the control in both cases (Fig. 1*c*). The similarity in chlorophyll quantities at 3 DAI between both bacterial treatments could be a consequence of disease symptoms produced by *Xcc* (Kraiselburd *et al*. 2013).

The 'response to stress' network includes genes related to abiotic stress

Inside the 'response to stress' category, four alleles similar to HSP genes were found: one HSP90 and two small HSP genes were repressed, and one HSP70 gene was induced (Fig. 2, Table S2). On the other hand, four alleles containing domains

similar to the DNAJ group were found: one was induced but the others three were repressed (Fig. 2, Table S2).

An important group of genes associated with abiotic stress that are also classified as 'response to stress' genes was found to exhibit expression changes in the *C. sinensis* nonhost response to *Xcv*, indicating a crossover between response pathways to different stresses in *Citrus* plants. These 28 genes could be classified into seven groups (Fig. 2, Table S2).

Also down-regulated were one allele with homology to UVB-resistance 8 protein (*UVR8* also known as regulator of chromosome condensation 1 or RCC1), one homolog to tumorous shoot development 2 (*TSD2*) and three homologs to dehydrin. Also, seven genes were similar to the universal stress proteins (USPs), four of which were repressed and three induced; six were similar to putative early dehydration-responsive (EDR) family proteins, five of which repressed and one induced; and three that were similar to wounding response proteins, which were downregulated.

In addition, seven alleles homologous to aquaporins exhibited modified expression, six repressed and one induced (Table S2). Phylogenetic clustering of *C. sinensis* domains with the *A. thaliana* aquaporins showed that five repressed *Citrus* alleles were grouped inside the plasma membrane intrinsic proteins (PIPs) subfamily and one was inside the tonoplast intrinsic proteins (TIPs) subfamily, and the induced one was in the NOD26-like intrinsic proteins (NIPs) subfamily (Fig. S2).

Participation of secondary metabolism

The analysis classified a set of genes involved in plant defence responses through the flavonoid biosynthetic pathways during the *C. sinensis* nonhost response to *Xcv* as 'secondary metabolism' genes. The expression of 14 alleles increased significantly, including core enzymes of the phenylpropanoid pathway such as one phenylalanine ammonia-lyase, two P450 reductase 2 and one 4-coumaroyl-CoA synthase-like; and nine enzymes directly involved in lignin biosynthesis such as two cinnamoyl CoA reductases (CCR), one caffeoyl-CoA 3-*O*-methyltransferase, one ferulate 5-hydroxylase, two hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferases and three caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (Table S3).

In order to confirm the expression patterns obtained in the microarray experiment for the secondary metabolism pathway, the regulatory enzyme of lignin biosynthesis, CCR1, was analysed by real-time PCR, revealing an induction pattern similar to that of the microarray data (Fig. 3). The lignin content was then analysed by staining leaf cross-sections at 48 h after infection and the increase observed, mainly in xylem cell wall thickness in response to *Xcv* in comparison to *Xcc* and the control (Fig. 4*a*, *b*), corroborated this modification. Also indicative of an increase in lignin deposition was a change from a spiral xylem structure in control and *Xcc* treatments to a scalariform structure in the *Xcv* treatment (Fig. 4*c*).

In addition, two alleles homologous to key enzymes of carotenoid biosynthesis, phytoene synthase and the zeta-carotene desaturase, were repressed, suggesting a decrease in the carotenoid contents. This diminution was confirmed by a significant reduction of carotenoids in *C. sinensis* leaves treated with *Xcv* in comparison to *Xcc* or the control at 1, 2 and 3 DAI (Fig. 5).

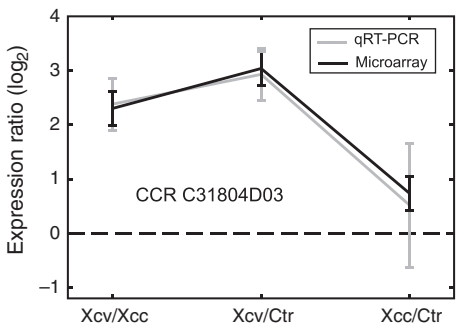

Fig. 3. Validation of microarray data by real-time reverse transcription–PCR (qRT-PCR) of the CCR allele in *Citrus sinensis*. The log_2 of the relative expression ratio between treatments are shown. The results are the mean of three replicates and error bars represent the s.e. Ctr, control; *Xcc*, *Xanthomonas citri* subsp. *citri*; *Xcv*, *Xanthomonas campestris* pv. *vesicatoria*.

Notably, eight differentially expressed alleles with similarity to GSTs were detected in this category, six of them induced and two repressed (Table S3). Phylogenetic clustering of *C. sinensis* and *A. thaliana* GST domains showed that five induced *Citrus* alleles grouped inside the GST-U subfamily and one induced inside the GST-F subfamily, and one repressed allele in the GST-Z subfamily (Fig. S3). Similar relationships were obtained using the complete sequences, including the other repressed allele (not detected previously because it does not have a GST *N*-terminal domain) inside the GST-L subfamily (data not shown). The expression pattern of one induced GST allele was analysed by real-time PCR, confirming the results obtained in the microarray assay (Fig. 6).

Discussion

The knowledge of plants' defence responses to pathogens constitutes an important scientific achievement and is also an essential step for recognising genes for plant disease control during breeding programs. In *Citrus* species, the major economically important fruit crops in the world, there is little information regarding the nonhost response to pathogens, the most general defence mechanism in plants. Previously we had characterised the *C. sinensis* nonhost response to the bacterium *Xcv* and we had obtained the plant's transcriptional profiling involved in this interaction (Daurelio *et al*. 2013). In this work, a new *in silico* analysis performed over this transcriptome allowed us to detect novel functional categories that were over-represented during this response, which were then confirmed through molecular or physiological assays. Real-time PCR assays of the microarray experiment were performed at the same times after treatment in order to validate the microarray data. The physiological studies were carried out at times after treatment that enabled the detection of the response, as discussed below for each analysis.

Although an improvement in the photosynthesis process in order to provide the energy required for plant defence may be expected, the repression of photosynthesis is a frequent host response to phytopathogens (Bolton 2009). In consequence,

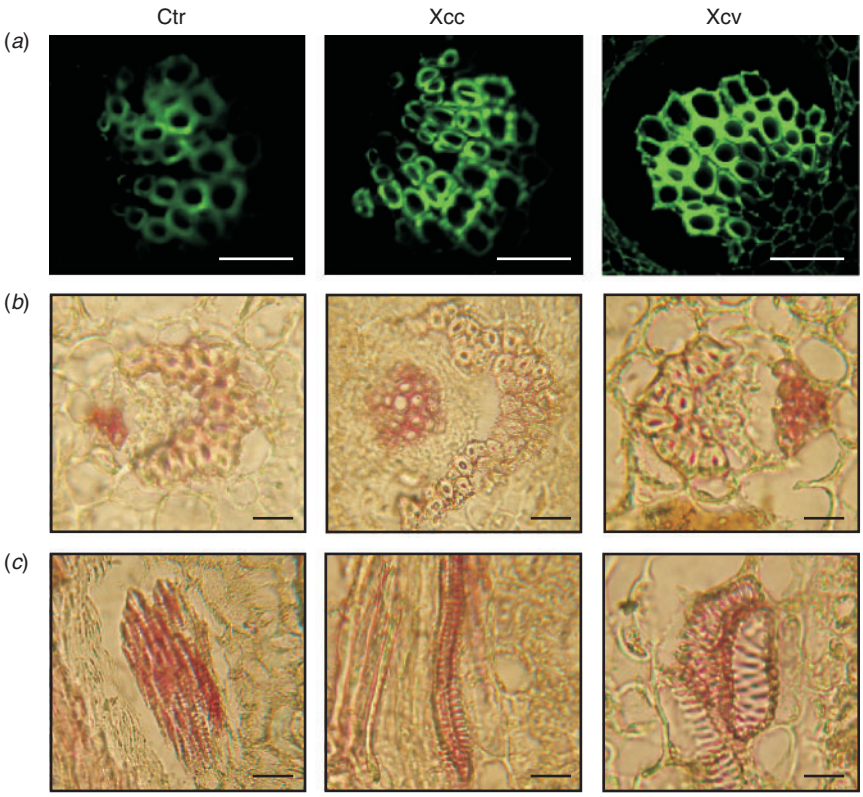

Fig. 4. Analysis of secondary metabolism during the nonhost response in *Citrus sinensis*. Histological analysis of lignin deposition by (*a*) acridine orange and (*b*, *c*) acid phloroglucinol staining in *C. sinensis* leaves infiltrated with *Xanthomonas citri* subsp. *citri* (*Xcc*) or *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) and control (Ctr) leaves at 48 h after inoculation (scale bars = 25 µm). Xylem vessels are observed in all panels (longitudinal in (*c*)); phloem and sclerenchymatous fibres surrounding the vascular bundles also are visualised in (*b*).

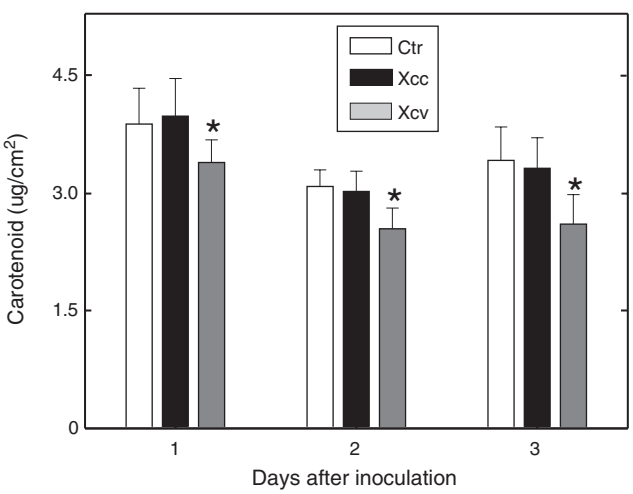

Fig. 5. Measurement of carotenoid content of bacteria-infected *Citrus sinensis* leaves. Treatments are indicated at the top of the figure. The results are the mean of three replicates and error bars represent the s.e. Mixed-model ANOVA and LSD multiple comparison tests were used for statistical analysis. Asterisks indicate significant differences between *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) versus *Xanthomonas citri* subsp. *citri* (*Xcc*) and control (Ctr) treatments ($P < 0.05$).

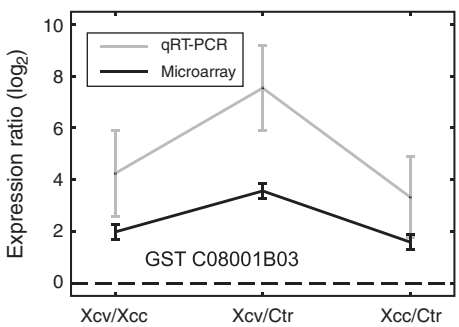

Fig. 6. Validation of microarray data by real time reverse transcription–PCR of a GST allele in *Citrus sinensis*. The $\log_2$ of the relative expression ratio between treatments are shown. The results are the mean of three replicates and error bars represent the s.e. Ctr, control; *Xcc*, *Xanthomonas citri* subsp. *citri*; *Xanthomonas campestris* pv. *vesicatoria*.

plants shift towards a nonassimilatory metabolism that involves a general downregulation of photosynthetic genes (Bilgin *et al.* 2010), with disruption of photosynthetic electron transfer and generation of ROS (Zou *et al.* 2005; Kangasjarvi *et al.* 2012). Accordingly, a reduction in photosynthesis during the *C. sinensis* nonhost response to *Xcv* was detected at the transcriptional

and physiological levels. The transcriptional repression of photosynthesis-related genes could be associated with the regulation of gene expression in response to changes in the redox state in the chloroplast (Zou *et al*. 2005). The decay of photosynthesis in plants is a mechanism that is highly dependent on the extension and timing of each plant–pathogen interaction (Kangasjarvi *et al*. 2012). In *Citrus*, physiological modifications were expected to occur later than the transcriptional changes and prior to the leaf structural disruption observed at 2 DAI (Daurelio *et al*. 2013); consequently, we analysed them at times spanning from 1 to 3 DAI. The decline in *C. sinensis* photosynthesis indicates the damage generated in the PSII complex (lower F_v/F_m), coupled with a decrease in electron transport and photochemistry (lower ϕ_{PSII} and maxϕ_{PSII}), and a reduction of plastoquinone pools (higher pressure excitation). The damaged PSII was still oxidised in the light and the over-reduction of the electron transfer chain promoted the donation of electrons to molecular O, leading to light-inducible ROS production in chloroplast stroma (Zou *et al*. 2005; Mur *et al*. 2010). Two mechanisms have been postulated to regulate ROS production and photodamage in the PSII. One involves the turnover of reaction centre D1 protein, which is degraded and replaced by *de novo* protein synthesis acting as a repair cycle. The specific downregulation of the chloroplastic FtsH proteases of D1, which were, in turn, repressed during the *C. sinensis* HR to *Xcv*, provides plants with a possible mechanism to elicit ROS production and cell death upon infection (Kangasjarvi *et al*. 2012). The other mechanism involves stay-green protein, which promotes chlorophyll degradation via disruption of LHCs (Mur *et al*. 2010). However, the two alleles of this gene in the *Citrus* microarray remained unchanged after Xcv. treatment (these genes were left off the list by cut-off restrictions), suggesting a slow or delayed induction of the *Citrus* HR in comparison with the *A. thaliana* HR (Mur *et al*. 2010). The reduction of photosynthesis produces a transition from source to sink in infected tissues, and carbohydrates and energy demand must be compensated for through an increase in different activities (Kangasjarvi *et al*. 2012). In *Citrus*, this compensation could be carried out by the induction of cell wall invertase and hexose transporters, including a homologue to the *STP13* allele, the expression of which in *A. thaliana* correlates with programmed cell death (Kloepper *et al*. 2004).

Although the turnover of the photosystem leads to chlorophyll release, a decrease in chl a content was observed, suggesting the diminution of chlorophyll biosynthesis or augmentation of the degradation pathways to produce the intermediates involved in the defence response through the generation of ROS in chloroplasts (Mur *et al*. 2010). The *Flu* allele is related to chlorophyll synthesis regulation as a negative feedback regulator of tetrapyrrole biosynthesis and *flu* mutants accumulate the chlorophyll precursor protochlorophyllide that leads to light-induced cell death (Kauss *et al*. 2012). The downregulation of the *Flu* and chlorophyll-haem biosynthetic enzymes suggests a deviation of the tetrapyrrole pathway to the sirohaem synthesis branch during the *Citrus* defence response (Kauss *et al*. 2012). *Flu* repression is mediated by two chloroplastic proteins, EXECUTER 1 and 2 (Mur *et al*. 2010), but they present two unchanged alleles that were left off the list by the cut-off restrictions. The *A. thaliana* Chlorophylase 1 initiates chlorophyll degradation and it is induced upon tissue damage, as observed in *C. sinensis* (Kangasjarvi *et al*. 2012). The downstream accumulation of the pheophorbide intermediate, in correlation with downregulation of its oxidase (pheophorbide a oxygenase, also called Accelerated Cell Death 1), has been associated with HR in *A. thaliana* (Mur *et al*. 2010). The next step was catalysed by red chlorophyll catabolite reductase (RCCR or Accelerated Cell Death 2), the repression of which was reported previously in the *C. sinensis* nonhost response (Daurelio *et al*. 2013). Altogether, the induction of Chlorophylase 1 and the repression of Flu, biosynthetic enzymes, ACD1 or ACD2 support the participation of chlorophyll metabolism intermediates during the *Citrus* nonhost response to *Xcv*. Additionally two early light-induced proteins alleles, which prevent photo-oxidative stress under light-stress conditions by transiently binding to free chlorophyll (Hutin *et al*. 2003), were repressed.

The differential expression of several HSPs suggests their participation in both types of stresses (biotic and abiotic) in *Citrus*. HSPs facilitate the growth and survival of plants under severe heat stress, allowing lethal temperature toleration for short periods, and also participate in *A. thaliana* defence processes against pathogens (Cheong *et al*. 2002). The repressed *C. sinensis HSP90* was similar to chloroplastic *HSP90.5* from *A. thaliana*, the overexpression of which produces a reduced tolerance of salt and drought stresses (Song *et al*. 2009). The induced *C. sinensis HSP70* showed homology to *A. thaliana HSP70T-2*, which is upregulated in these stress responses (Rizhsky *et al*. 2004). The induction of sHSPs during plant–bacteria interactions was observed in tobacco (*Nicotiana tabacum* L.), but none matched with the *Citrus* alleles and they were repressed (Maimbo *et al*. 2007). The participation of the conserved and essential DNAJ and HSP40 proteins in plant–virus interactions has been reported (Lu *et al*. 2009), whereas the differential expression detected for the *Citrus* alleles hints their participation during the defence response to bacteria.

The detection of several genes related to abiotic stress provides evidence of stress network crosstalk in *Citrus*. The *Citrus* dehydrin Cold-regulated 15 interacts with DNA, supposedly to protect it in plant cells during seed maturation and stress responses (Hara *et al*. 2009); thus the repression observed could allow the exposition of DNA to endonucleases for the typical degradation observed during HR. A similar function could be suggested for the *C. sinensis* UVR8 repression, a UV-B-specific signalling component that orchestrates the expression of several genes with vital UV protective functions through association with chromatin through histones (Cloix and Jenkins 2008).

The EDR, wounding response and USP families do not have a defined function in the defence response as yet. However, as one exception, the *A. thaliana* EDR15 protein, which was rapidly induced in response to stress, was proposed as a novel mediator of stress-related ABA signalling in *A. thaliana* (Kariola *et al*. 2006), but the only induced *C. sinensis* allele was homologous to *A. thaliana* EDR4. The *C. sinensis* wounding response alleles were all repressed, including one that was similar to the *A. thaliana AT4G10270*, which was strongly upregulated by potassium deficiency, and one that was similar to a tomato (*Solanum lycopersicum* L.)

wound-inducible gene (Shin and Schachtman 2004). The USP family has no assigned function in plant stress (Kerk *et al*. 2003) and the *Citrus* alleles showed diverse patterns of regulation.

Aquaporins, also called major intrinsic proteins in higher plants, facilitate water transport over cellular membranes, playing an important role in water homeostasis (Alexandersson *et al*. 2010). The major repression of aquaporins observed during the soybean (*Glycine max* (L.) Merr.) HR to *Pseudomonas syringae* (Zou *et al*. 2005) and during the *C. sinensis* HR to *Xcv* adds to a mostly downregulated mechanism for these proteins. This repression could help to reduce photosynthesis rates and help to diminish bacterial growth by keeping water from the apoplast (Zou *et al*. 2005). However, some aquaporins facilitate hydrogen peroxide transport during essential processes in plants, including defence against pathogens (Dynowski *et al*. 2008). Noticeably, the only induced aquaporin in the *Citrus* response to *Xcv* was orthologous and phylogenetically related to one of these alleles from *A. thaliana* (*NIP1;2*, *AT4G18910*).

The participation of genes involved in the phenylpropanoid pathway has been reported during biotic stress in different plants (Xu *et al*. 2011), as well as in the induction of resistance in *Citrus* fruits challenged with elicitors (Ballester *et al*. 2011). Transcriptome modifications during the *C. sinensis* nonhost response to *Xcv* identified genes of the main phenylpropanoid pathway that lead to the synthesis of lignin. Lignin deposition was evaluated at 2 DAI because it was estimated to occur later than induction of the lignin biosynthetic genes and possibly at the same time as the observed leaf structural disruption (Daurelio *et al*. 2013). The results obtained are in agreement with previous studies that showed lignin pathway activation during incompatible interactions, since lignification and reinforcement of cell walls constitute important processes in the response of plants to pathogen attack (Naoumkina *et al*. 2010). A decrease in the carotenoid content, which was also involved in the secondary metabolism, was shown to occur as part of the *Citrus* nonhost response to bacteria, as previously reported in the disease process during the tomato–pepino mosaic virus interaction (Hanssen *et al*. 2011). The time points for carotenoid quantification were selected by applying the same criterion used for physiological photosynthesis changes.

GST proteins catalyse the addition of glutathione to a variety of substrates, mainly during the cellular detoxification process (Lieberherr *et al*. 2003), and play a significant role in the reduction of damage caused by pathogens by limiting the extent of cell death during HR (Lieberherr *et al*. 2003). The induction of GSTs by pathogens has been reported, although the signalling pathways are not well understood (Lieberherr *et al*. 2003). The high number of upregulated GSTs indicates their participation during the *C. sinensis* HR to *Xcv*, where five alleles belong to the GST-U subfamily, which are novel regarding the GST-F alleles induced during the defence response in *A. thaliana* (Lieberherr *et al*. 2003).

The knowledge of plant nonhost defence mechanisms, which are yet to be fully understood, is necessary to succeed in breeding crops for long-term disease resistance (Senthil-Kumar and Mysore 2013). The novel aspects presented in this work, in association with those previously described (Daurelio *et al*. 2013), contribute to the overall comprehension of the *Citrus* nonhost response (Fig. S4). On the basis of our results, we propose that accumulation of ROS in the apoplast may be triggered by plasma membrane-bound NADPH oxidases in early stages of the plant response, supported by the induction of the *RBohD* allele (Daurelio *et al*. 2013). Chloroplasts would then contribute to ROS production through photosystem disruption and chlorophyll degradation, and induced aquaporins would participate in their distribution. The electrolyte leakage and tissue disruption produced by *Xcv* (Daurelio *et al*. 2013) agree with photosynthesis energy dissipation in a nonregulated way that is distinctive for tissue injury and the repression of TSD2 methyltransferase, which is involved in cell adhesion (Krupková *et al*. 2007). Moreover some evidence indicates that light-dependent defence and death signals could originate from a reduction of the plastoquinone poo, and then be relayed to the nucleus by means of cytosolic components as Phytoalexin Deficient 4 (Kangasjarvi *et al*. 2012), a central regulatory node in plant immunity upregulated in the *Citrus* nonhost response (Daurelio *et al*. 2013). The HSP expression changes could be regulated by heat stress factors (HSFs) transcription factors (Daurelio *et al*. 2013). Furthermore, the antioxidant activity of GST proteins could participate in the attenuation of HR symptoms (Daurelio *et al*. 2013). This multiplicity of pathways agrees with the description of the nonhost response as being governed by a broad range of multilayered mechanisms regulated by numerous genes (Senthil-Kumar and Mysore 2013). Nevertheless, it is not possible to consider any one of them as the cause of the *Citrus* nonhost response. In conclusion, the results presented in this work add considerable knowledge about the nonhost defence in *Citrus*, trees species and nonmodel plants, even though the establishment of the initial causes of this response will require further study.

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