Phytohormone participation during *Citrus sinensis* non-host response to *Xanthomonas campestris* pv. *vesicatoria*

Silvana Petrocelli[a], María D. Pizarro[b,c], Analía Alet[a], Carlos De Ollas[d], Manuel Talón[e], Francisco R. Tadeo[e], Aurelio Gómez-Cadenas[d], Vicent Arbona[d], Elena G. Orellano[a,c,f], Lucas D. Daurelio[b,c,*]

[a]Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina.

[b]Laboratorio de Investigaciones en Fisiología y Biología Molecular Vegetal (LIFiBVe), Cátedra de Fisiología Vegetal, Facultad de Ciencias Agrarias, Universidad Nacional del Litoral, Esperanza, Argentina.

[c]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

[d]Departament de Ciències Agràries i del Medi Natural, Universitat Jaume I, Castelló de la Plana, Spain.

[e]Centre de Genómica, Institut Valencià d'Investigacions Agràries (IVIA), Montcada, València, Spain

[f]Instituto de Biología Molecular y Celular de Rosario (IBR), Rosario, Argentina.

*Correspondence: Lucas D. Daurelio, Laboratorio de Investigaciones en Fisiología y Biología Molecular Vegetal (LIFiBVe), Cátedra de Fisiología Vegetal, Facultad de Ciencias Agrarias, Universidad Nacional del Litoral, Kreder 2805 (S3080HOF), Esperanza, Santa Fe, Argentina. Tel: +54 341 6213826.

E-mail: lucasdaurelio@gmail.com

Abstract

Citrus, one of the most valuable fruit crops around the world, are severely damaged by biotic stress and huge economical losses are caused by pathogen infections. Non-host response is an essential plant defense mechanism against pathogen attack, however is still not completely characterized and is poorly studied in non-model plants. In previous reports, we characterized *C. sinensis* non-host response to *Xanthomonas campestris* pv. *vesicatoria* (Xcv), in comparison to infection caused by *Xanthomonas citri* subsp. *citri* (Xcc). This was described as a hypersensitive response with structural and physiological modifications, and transcriptional reprogramming of pathogen related proteins and transcription factors, among others. Phytohormones serve as key regulators in plant response to stress, by means of interconnected complex pathways. Here, we study the participation of phytohormone pathways during Citrus non-host response to Xcv. Our results indicate a decrease in abscisic acid, cytokinins, gibberellins, brassinosteroids, auxins and nitric oxide, an increase in ethylene and salicylic acid, constant levels of jasmonic acid, whereas polyamine levels change in a very specific pattern. The present work provides a first broad approach to hormone participation during non-host response in a non-model plant of the *Citrus* genus, also representative of woody plants.

Key words: biotic stress; incompatible interaction; plant - pathogen interaction; sweet orange; transcriptomic analysis; *Xanthomonas citri* subsp. *citri*

1. Introduction

Plants are constantly exposed to biotic stress. The result of this plant - pathogen initial contact can be classified as compatible, resulting in plant disease, or incompatible, when the plant becomes resistant to pathogen invasion. The outcome depends on several plant defense strategies triggered through pathogen invasion. During plant invasion, physical and chemical constitutive barriers restrict pathogen entry and infection. In addition, a wide variety of inducible defense mechanisms are initiated upon pathogen recognition by pathogen-associated molecular patterns (PAMPs). This basal resistance is called PAMP-triggered immunity (PTI) (Senthil-Kumar and Mysore, 2013). In the co-evolution of pathogens and their host plants, pathogens have acquired the ability to suppress PTI by delivering

effector molecules into the plant cell that promote pathogen growth and disease, producing an effector-triggered susceptibility. In turn, plants have developed resistance proteins that recognize specific effectors resulting in a secondary immune response known as effector-triggered immunity or ETI (Senthil-Kumar and Mysore, 2013).

Non-host resistance is considered an incompatible interaction; it is a general mechanism that involves a broad-spectrum defense against all isolates of a potential pathogen and is the most common form of plant resistance to pathogenic microorganisms (Senthil-Kumar and Mysore, 2013). The nature of the defense mechanisms activated in plants during non-host response is not completely understood but it is believed that both constitutive and inducible reactions are involved (Senthil-Kumar and Mysore, 2013).

Plant defense against several pathogens produces changes in the levels of various phytohormones (Adie et al., 2007;Verma et al., 2016). Phytohormones are compounds with diverse chemical structures that regulate numerous aspects of plant growth, development and response to abiotic and biotic stresses, functioning in a complex signaling network (Verma et al., 2016). They are grouped into different classes that exert characteristic biological effects, though their responses are often mediated by interrelated actions in signaling crosstalk, for example, during organ development and plant response to different stresses (Verma et al., 2016). Auxins, gibberellins (GA), cytokinins (CK), abscisic acid (ABA), ethylene (ET), salicylic acid (SA), jasmonates (JA), brassinosteroids (BR), nitric oxide (NO) and strigolactones are among the most important phytohormones (Verma et al., 2016). In addition, the inclusion of polyamines (PAs), like Putrescine (Put), Spermidine (Spd) and Spermine (Spm), into this group is under debate, since there are many aspects of PAs function and regulation that resemble those of phytohormones, such as their involvement in organogenesis, embryogenesis, and abiotic and biotic plant stress responses (Arbona and Gómez-Cadenas, 2008; Jiménez Bremont et al., 2014).

Among the above mentioned compounds, mainly ABA, SA, JA and ET have been involved in regulating plant defense against abiotic and biotic stresses (Verma et al., 2016). In particular, a complex regulatory network between the phytohormones JA, SA and ET has been shown to regulate the signal transduction pathways activated during non-host defense response (Bari and Jones,

2009;Verma et al., 2016). In addition, other phytohormones, including ABA, GA and CKs have been involved in the regulation of plant defense response (Verma et al., 2016). However, the molecular mechanisms of each hormone pathway induced during defense responses are poorly understood (Bari and Jones, 2009). On the other hand, pathogens can counteract the plant response by producing changes in phytohormone homeostasis for their own benefit (Chen et al., 2007).

Citrus species, one of the major fruit crops worldwide, are seriously affected by abiotic and biotic stresses and devastating losses are caused by pathogen infections (Talon and Gmitter, 2008). Therefore, the study of the defense mechanisms induced by Citrus plants during biotic stress could help to prevent these economical losses. Formerly, the incompatible interaction of *C. sinensis* (sweet orange) leaves with the Gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* (Xcv) was characterized as an non-host Hypersensitive Response (HR) employing biochemistry assays and transcriptomic analysis (Daurelio et al., 2013). In the mentioned study, Citrus canker, one of the most destructive Citrus diseases caused by *Xanthomonas citri* subsp. *citri* (Xcc) (Brunings and Gabriel, 2003), was used as disease control. The global expression profile of *C. sinensis* response to Xcv allowed the identification of several differentially expressed genes as part of the over-represented categories during the induced resistance in Citrus plants (Daurelio et al., 2009; 2013; 2015). Additionally, recent proteome analysis of Citrus during the non-host response to *Xanthomonas oryzae* pv. *oryzae* revealed novel proteins differentially regulated in the nucleus and the extracellular matrix in comparison to Xcc infection (Rani and Podile, 2014;Rani et al., 2015).

Modulation of hormonal responses, in which PAs were included as phytohormones, has been observed in different species of *Citrus* genus during the abiotic stress caused by soil flooding (Arbona and Gómez-Cadenas, 2008). Nonetheless, the participation of phytohormone pathways during biotic stress, particularly during non-host response, in Citrus plants has not been reported so far. Therefore, the aim of the present work was to analyze hormone participation in *C. sinensis* non-host response to Xcv, to give a particular insight of this response mechanism in Citrus and discover similarities with the non-host response in other plants. The results presented here indicate a complex participation of phytohormones, including non-conventional results to that observed in model plants, and the possible mechanisms are herein discussed. In addition, the present work provides a first broad approach to

hormone participation during non-host response in a non-model plant of the *Citrus* genus, also representative of woody plants.

2. Materials and Methods

2.1. Plant material, bacterial strains and plant inoculation

Citrus sinensis cv. Valencia Late plants gently provided by Catalina Anderson (INTA Concordia, Argentina) were grown in greenhouse at 25/18°C (day/night temperatures) with a 14 h photoperiod (150 $\mu E/m^2s$) and controlled relative humidity. Young fully expanded leaves (one month old approximately) were used in all experiments.

X. campestris pv. *vesicatoria* (Doidge, Xcv) and *X. citri* subsp. *citri* (Hasse, Xcc) strains were routinely grown aerobically in Silva Buddenhagen (SB) medium (Daurelio et al., 2009) at 28ºC with shaking at 200 rpm, or on 1.5% (w/v) SB-agar plates, supplemented with ampicillin 25 µg/ml for Xcc. The abaxial side of leaves was infiltrated by pressure with 10^7 colony forming units/ml of bacterial suspensions (Xcc and Xcv) or with the carrier used to inoculate the different bacteria, 10 mM $MgCl_2$ (control, Ctr), using a syringe without a needle (Daurelio et al., 2009).

2.2. Microarray data acquisition and analysis

In order to identify hormonal pathways involved in the non-host response of *C. sinensis* to Xcv, the transcriptomic data from leaves treated with Xcv, Xcc as disease control and the carrier solution as negative control were analyzed (Daurelio et al., 2013). Microarray data for Citrus response to Xcv in comparison to Xcc and Ctr was obtained from Daurelio *et al.* (2013). Differences in gene expression were considered significant when q-values (p-values corrected for Hochberg false discovery rate or FDR) were lower than 0.05 and the cutoff for M value ($\log_2$ of expression ratio between treatments) was ± 0.6, indicating 50% change in relative expression. Differentially expressed genes between Xcv-Xcc and Xcv-Ctr were analyzed to identify biological processes related to hormone metabolism that were differentially regulated. A Singular Enrichment Analysis (SEA) was used, with the hypergeometric statistical test and Hochberg FDR correction, by means of the Web-based platform agriGO - GO Analysis Toolkit and Database for Agricultural Community (Du et al., 2010).

141

142 *2.3. Determination of plant hormone levels*

143 Control and treated leaves were harvested 12 hours post infection (hpi), immediately frozen in liquid
144 nitrogen, ground to a fine powder and lyophilized (-50° C, 6 to 8 h). This time post infection was
145 selected because the hormone level changes should be posterior to the transcriptional modifications
146 analyzed at 8 hpi. Three biological replicates of leaves taken from three independent plants were
147 prepared and independently processed. SA, ABA, and JA were analyzed by UPLC coupled to tandem
148 mass spectrometry (Durgbanshi et al., 2005). Lyophilized tissue (0.5 g) was directly weighed and
149 extracted in ultrapure water using a tissue homogenizer (Ultra-Turrax, Ika-Werke, Staufen, Germany).
150 Before extraction, 50 µl of a mixture of internal standards containing 50 ng of d6-ABA, 50 ng of
151 dihydrojasmonic acid and 50 ng of d6-SA acid were added to assess recovery and matrix effects
152 (Arbona and Gómez-Cadenas, 2008). After extraction and centrifugation, the pH of the supernatant
153 was adjusted to 3.0 and partitioned twice against diethylether. The organic layers were combined and
154 evaporated in a centrifuge vacuum evaporator. The dry residue was thereafter resuspended in a
155 water:methanol (9:1) solution, filtered, and injected into a UPLC system (Acquity SDS, Waters Corp.,
156 Milford, MA). Hormones were then separated on a reversed-phase Gravity column (Macherey-Nagel,
157 50 x 2.1 mm 1.8-µm particle size) using methanol and water as solvents, both supplemented with 0.1%
158 acetic acid, at a flow rate of 300 µl/min. The mass spectrometer, a triple quadrupole (Xevo TQD,
159 Micromass Ltd., Manchester, UK), was operated in negative ionization electrospray mode and the
160 different plant hormones were detected according to their specific transitions using a multiresidue
161 mass spectrometric method (Durgbanshi et al., 2005).

162

163 *2.4. Polyamines analysis*

164 Control and treated leaves were harvested at 0, 8, 12 and 24 hpi, immediately frozen in liquid nitrogen,
165 ground to a fine powder and lyophilized (-50° C, 6 to 8 h). These times post infection were selected
166 because the hormone levels changes should be posterior to the transcriptional modifications analyzed
167 at 8 hpi, which was included in this case. Three biological replicates of leaves taken from three
168 independent plants were prepared and independently processed. PAs were determined as dansyl

chloride derivatives according to Hunter (1998) following protocol of Arbona and Gómez-Cadenas (2008). Tissue (0.4 g) was extracted in 10% $HClO_4$ (Panreac) using a tissue homogenizer (Ultra-Turrax, Ika-Werke, Staufen, Germany). After centrifugation at 4ºC to pellet debris, 200 µl of the supernatant were combined with 200 µl of a saturated $NaHCO_3$ solution and 400 µl of a 5 mg/ml dansyl chloride solution (Fluka, Buchs, Switzerland) in ice-cold acetone. Samples were incubated at 70ºC in a water bath for 10 min and subsequently allowed to cool down at room temperature. Afterward, 100 µl of a 100 mg/ml solution of proline (Panreac) were added as a quencher for dansyl chloride and incubated in the dark at room temperature for 30 min. Then, extracts were partitioned against 500 µl of toluene (Panreac) that were recovered and evaporated in a centrifuge vacuum evaporator (Jouan) at room temperature. The dry residue was resuspended in 800 µl of acetonitrile (Scharlab) and filtered through 0.22 µm cellulose acetate filters prior to injection into a HPLC system (Agilent 1100 Series, Agilent Technologies Ltd., Palo Alto, CA). Samples were separated in a C18 column (Kromasil 100, 5 µm, 146 x 4.6, Scharlab) at a flow rate of 1.5 ml/min using an acetonitrile:water gradient. Derivatized Put, Spd and Spm were detected by fluorescence. A constant amount of 1,5-diaminoheptane (Sigma-Aldrich) was used as an internal standard to normalize peak areas.

2.5. RNA isolation and Real time RT-PCR

Control and treated leaves were collected at 0, 8 and 24 hpi. Three biological replicates of leaves taken from three independent plants were prepared and independently processed. Tissue was harvested in liquid nitrogen and stored at -80ºC 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. Primers were designed using Primer3 v.0.4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Rozen and Skaletsky, 2000). The alleles analyzed, primer sequences and product lengths are indicated in Supplementary Material (Table S1). One microgram of total RNA was used for cDNA synthesis with the M-MuLV Retro Transcriptase enzyme (Promega, USA) and d(T)22 oligonucleotide, following the manufacturer's instructions. Real time RT-

196 PCR reactions were carried out in StepOne real-time PCR system (Applied Biosystems, USA)
197 equipped with StepOne™ Software v2.2.2. Reactions were performed with 1 µl of cDNA template and
198 a SYBR green-I reaction mixture containing 1:50,000 diluted SYBR green-I (Invitrogen), 10 pmol of
199 each primer, 0.5 U Platinum-Taq DNA polymerase (Invitrogen), 40 mmol dNTPs, 3.75 mM $MgCl_2$
200 and 1X Platinum-Taq buffer in a final volume of 20 µl under the following conditions: 95ºC for 1 min
201 followed by 40 cycles of 95ºC for 15 s, 59ºC for 20 s and 72ºC for 40 s. Fluorescent intensity data was
202 acquired during the 72ºC extension step. Specificity of the amplification reactions was assessed by
203 melting curve analysis, which were run at 95ºC for 15 s and 60ºC for 15 s followed by an increase in
204 temperature from 60 to 85ºC (0.2ºC/s) with continuous fluorescence recording. PCR reactions without
205 the reverse transcription step did not yield products. In addition, real time PCR products using
206 genomic DNA or cDNA templates for the actin housekeeping gene were sized differently, allowing
207 the detection of genomic DNA contamination. To perform the analysis of relative expression, we used
208 the $2^{-\Delta\Delta CT}$ method , where ΔCt represents the difference between Ct (cycle threshold) values of a target
209 and the endogenous control (actin) in the same sample, and ΔΔCt is the difference between the ΔCt
210 value of a particular sample and the mean of ΔCt of control samples used as reference (Daurelio et al.,
211 2013).
212
213 *2.6. Statistical and phylogenetic analysis*
214 ABA, JA and SA hormone quantifications were statistically analyzed using a non-parametric
215 Friedman test, considering plants as blocks, followed with ranks sum multiple comparisons. PAs
216 quantifications were analyzed using a two-factor (treatment and time pi) mixed model ANOVA and
217 DGC multiple comparison tests along with residual analysis and validation. Real time RT-PCR results
218 were analyzed using One-way ANOVA and Bonferroni multiple comparison tests along with residual
219 analysis and validation. Differences were considered statistically significant for p-values < 0.05.
220 Due to the complexity of PA pathways, *C. sinensis* and *C. clemenules* genes involved in the
221 biosynthesis and degradation of PAs were identified by comparison with the sequences deposited in
222 the database using Phytozome (www.phytozome.net). For ornithine decarboxylase (ODC), which is
223 not present in *Arabidopsis*, sequences from *Oryza sativa* and *Glycine max* were used. Phylogenetic

trees for PAs genes pathways (5,000 bootstrap) based on the Neighbor–Joining method were generated with the MEGA4 program as previously described (Daurelio et al., 2013).

3. Results and Discussion

3.1. Hormone related processes are differentially regulated during Citrus non-host response to Xcv

Transcriptomic data analysis showed a total of 3474 putative unigenes in the Citrus cDNA microarray that changed their expression levels in response to Xcv, in comparison to Xcc and Ctr, and were considered candidates to be involved in Citrus non-host response (Supplementary Fig. S1, intersection between Xcv-Ctr and Xcv-Xcc). Out of these, 2561 were successfully categorized in agriGO platform. SEA revealed that “response to hormone stimulus”, “hormone-mediated signaling pathway”, “cellular response to hormone stimulus”, “regulation of hormone levels” and “hormone metabolic processes” functional categories were over-represented during *C. sinensis* non-host response to Xcv (Supplementary Table S2). Among these hormone-related categories, "response to hormone stimulus" showed the highest representation, with 122 genes that changed their expression. Therefore, this over-representation of hormone related biological processes is indicative of their participation in *C. sinensis* non-host response to Xcv.

Genes involved in phytohormones metabolism and signaling were searched to gain insight into the role hormones play in non-host response and how they do it, and a total of eighty hormone-related genes were analyzed (Fig. 1 to 5, Supplementary Table S3). In addition, the endogenous levels of ABA, JA, SA and PAs, previously reported to be involved in plant stress responses, were determined in *C. sinensis* leaves inoculated with Xcv and Xcc strains (Fig. 1 to 5). The results obtained and the possible roles played by the different hormones are presented and discussed in the next sections.

3.2. ABA levels decrease as part of Citrus non-host response

ABA has long been associated with plant response to abiotic stress, as evidenced by increased levels of this hormone in plants exposed to heat, drought and salinity, among other environmental conditions (Verma et al., 2016), though a possible role in biotic stress has emerged (Cao et al., 2011). In the transcriptomic analysis performed herein, eleven ABA related genes were found to be differentially

252 expressed, indicating ABA diminution during Citrus-Xcv interaction (Fig. 1A and B, Supplementary
253 Table S3). First of all, two alleles of zeaxanthin epoxidase (*ABA1/ZEP*) and nine-cis-epoxycarotenoid
254 dioxygenase 4 (*CCD4*), enzymes that catalyze the first and a key regulated step of ABA biosynthesis
255 respectively, were down-regulated (Priya and Siva, 2015). In addition, the genes that encode ABA-
256 induced proteins HVA22E and ABA responsive elements-binding factor (*ABF3/DPBF5*) were also
257 repressed (Chen et al., 2002;Fujita et al., 2005). This strong decrease in ABA levels suggested by
258 transcriptomic analysis was confirmed through direct quantification of the hormone in *C. sinensis*
259 leaves during non-host response to Xcv (Fig. 1C).
260 Also, in correlation with ABA decrease, a strong repression of ABA-induced outer membrane
261 tryptophan-rich sensory protein coding gene (*TSPO*) was observed (Vanhee et al., 2011). It has been
262 postulated that TSPO binds porphyrins, allowing their degradation through an autophagy-dependent
263 mechanism (Vanhee et al., 2011). This lower level of TSPO leaves porphyrins available to continue
264 with the tetrapyrrole pathway deviation to the sirohaem synthesis branch, as was previously postulated
265 to occur during *C. sinensis* non-host response (Daurelio et al., 2015).
266 Therefore, our results indicate a clear decrease in ABA as part of the non-host response triggered after
267 challenging *C. sinensis* with Xcv. The observed induction of the genes encoding for some GRAM
268 domain ABA-induced proteins (GEM, GRE5 and GRE8) could be due to their responses to other
269 regulators (Mauri et al., 2016).
270
271 *3.3. Citrus non-host response can be associated to SA-ET positive signaling and absence of JA*
272 *participation*
273 Different studies performed with plants exposed to pathogen infections have shown increased levels of
274 SA, JA and ET, pointing out these phytohormones as crucial elements in the regulation of plant
275 defense against biotic stress. Particularly, SA seems to be responsible for defense against biotrophic
276 and hemi-biotrophic pathogens, while ET and JA usually mediate the response to necrotrophic
277 pathogens and herbivorous insects attack (Verma et al., 2016).
278 In our study, five up-regulated genes of the SA pathway were detected in Citrus non-host response
279 (Fig. 2A, Supplementary Table S3). Mainly, two alleles of glutaredoxin C9 (*GRX480/GRXC9/GRX8*)

presented induction. These encode SA-induced glutaredoxin that interacts with TGA-TFs to induce SA responsive genes and to repress JA responsive genes (Ndamukong et al., 2007). The lack of significant differences in the levels of free SA measured in leaf samples treated with Xcv, Xcc or Ctr (Fig. 2B) could be due to the synthesis of different SA derivatives, as was described in *Arabidopsis* non-host response (Mishina and Zeier, 2007). This fact is supported by the induction of SA UDP-glucosyl transferase gene (*UGT74F1*), a rapidly induced gene in response to pathogens in *Arabidopsis* (Song, 2006), and the gene that encodes for a putative benzoate-SA methyl transferase. Besides, SA participation in Citrus non-host response is also implied by the high induction of the SA-associated markers *PR1* and *PR5*, and the main SA signaling gene, *PAD4*, as previously reported (Daurelio et al., 2013).

The expression patterns of thirteen genes related to JA indicate a decrease in pathways related to this hormone during Citrus response to Xcv (Fig. 3A and B, Supplementary Table S3). Above all, the induction of the gene coding for the JA-signaling repressor jasmonate-Zim-domain 1 protein (*JAZ1/TIFY10A*) should attenuate JA mediated signaling (Bari and Jones, 2009). The peroxisomal 3-ketoacyl-CoA thiolase 3 gene (*KAT2/PED1*), that encodes for an enzyme involved in three steps of JA biosynthesis, was down-regulated. Finally, the up-regulated expression of benzoato-JA methylase-like C7A10.890 gene would favor the formation of Me-JA, postulated as a biologically inactive derivative (Wu et al., 2008). When the amount of JA was measured, no difference was found between Xcv and Ctr treatments, while inoculation with Xcc showed a 2-fold increased with respect to Xcv and Ctr (Fig. 3C). These results confirm that JA is not involved in Citrus non-host response to Xcv, while the rise in Citrus canker should be explored.

Finally, the expression pattern of three genes related to ET metabolism and five others that showed homology with typical ET signaling genes (Fig. 4, Supplementary Table S3) denote this hormone participation during *C. sinensis* non-host response. These include the induction of the genes coding for the synthesis enzyme, ACC oxidase or ET forming enzyme (*ACO4*) (Eckert et al., 2014), and for the components of ET downstream signaling pathway, *MKK4*, *MKK9*, *MAPK3* and ET insensitive 3 (*EIN3*) (Chen et al., 2009), with repression of the JA-ET signaling negative regulator cellulose synthase gene (*CESA3*) (Ellis et al., 2002). Other induced genes were constitutive triple response 1

(*CTR1*), a regulator of ET response (Hall et al., 2012), and N-acyltransferase Hookles 1 (*HLS1/COP3*), a target gene of the ET-activated transcription factor EIN3 (Zhang et al., 2014).

In agreement with the results here described, Adie *et al.* (2007) demonstrated that ABA is essential for JA biosynthesis during Arabidopsis defense response and Mishina and Zeier (2007) established that an increase in SA and SA derivatives with constant JA level were observed in Arabidopsis non-host response to bacteria. Furthermore, it has been informed that ABA and SA signaling pathways appear to be predominantly antagonistic (Cao et al., 2011).

3.4. Auxins, BR, CK, GA and NO levels decrease during Citrus non-host response

Besides the above mentioned hormones, recent studies have shown that auxins, GAs and CKs could also be involved in regulating plant stress response, particularly through a crosstalk mechanism with ABA, SA, JA and ET (Verma et al., 2016). In this work, we have analyzed the expression patterns of auxins, BR, CK, GA and NO to establish their roles in Citrus response to Xcv.

As part of their basal defense, plants reduce auxins levels because these compounds may facilitate pathogen invasion (Chen et al., 2007). The expression patterns observed for the differentially expressed genes (DEG) associated to auxins correlate with these hormones diminution (Fig. 4, Supplementary Table S3). The repression of the genes coding for two IAA-aminoacid hydrolases (*ILL3* and *ILL4*) and the induction of IAA-amido synthase gene (*VAS2/GH3.17*), should give rise to the conjugated-inactive form of indole-3-acetic acid (IAA) (Korasick et al., 2013). In turn, the dominant auxin receptor gene, that encodes for auxin signaling F-box 2, that is negatively regulated by the flagellin-induced miR393a during Arabidopsis bacterial resistance (Parry et al., 2009), showed repression. Also, small auxin up-regulated RNAs (*SAUR*), induced by auxins (Markakis et al., 2013), were mostly repressed, while induction of auxin-regulated gene involved in organ size (*ARGOS*) may occur due to ET increase (Markakis et al., 2012). Auxins activity is also diminish through the repression of the gene coding for auxin efflux carrier PIN-LIKES 2 (*PILS2*), a protein that regulates intracellular IAA compartmentalization and thus IAA availability for nuclear auxin signaling (Barbez et al., 2012), and the suppressor of auxin resistance 1 gene (*SAR1/NUP160*), affecting the transport of auxin response proteins (Parry et al., 2006).

The role of BR in plant defense was recently assessed in host responses, promoting a cell wall-based defense (Marcos et al., 2015), but not in non-host response. Our analysis identified five genes related to BR pathways differentially expressed during Citrus response to Xcv that indicate a reduction in BR level during non-host response (Fig. 4, Supplementary Table S3). The genes coding for the essential proteins for BR synthesis, cell elongation protein DIMINUTO (*DIM*) and constitutive photomorphogenic DWARF (*DWF3*), were repressed (Du and Poovaiah, 2005;Zhiponova et al., 2013), while the BR inactivating DON-Glucosyltransferase 1 gene (*DOGT1*) was induced (Poppenberger et al., 2005). On the other hand, the down-regulation of *BAS1*, that codes for PHYB activation tagged supressor 1, which inactivates BRs by hydroxylation, should be a consequence of BR-responsive transcription factor gene (*BZR1*) down-regulation, as BZR1 activates *BAS1* expression (Youn et al., 2016). Noticeably, BRs reduction coincides with auxins decrease regulated through ARF7 (Youn et al., 2016).

With regard to CK metabolism, five related genes were found to be differentially expressed, out of which two suggest a reduction in CK signaling during Citrus non-host response (Fig. 4, Supplementary Table S3). The gene that encodes for Zeantin CK-inactivating enzyme (*UGT76C2*) was induced (Hou et al., 2004), while the one coding for a positive regulator of CK signaling, the Histidine-containing phosphotransferase 5 (*AHP5*), presented down-regulation (Hutchison et al., 2006). It is known that CK up-regulates plant immunity via the induction of SA-dependent defense responses, whereas SA, in turn, inhibits CK signaling (Argueso et al., 2012). Our results provide evidence of the occurrence of this last inhibition stage in Citrus response to Xcv.

Concerning GA metabolism and signaling, four genes were differentially regulated as part of Citrus non-host response (Fig. 4, Supplementary Table S3). The gene coding for the GA-receptor insensitive DWARF1C (*GID1C*) (Nakajima et al., 2006) presented repression, while the ones that encode for the catabolic GA 2-oxidase (*GA2OX2*) and the cytochrome P450 involved in the inactivation of early GA intermediates (*CYP714A1*) (Yamauchi et al., 2007;Zhang et al., 2011) were induced. Their expression patterns indicate GA reduction. Additionally, the down-regulation of the coding gene for the signaling negative regulator DELLA belonging to the GRAS family protein (*GAI*), which is induced by GA and,

in turn, suppress SA response, agrees with the activation of the different SA-dependent pathways observed in Citrus response to Xcv (Navarro et al., 2008).

The transcriptomic analysis allowed identifying five genes related to NO metabolism that indicate a diminution in NO content (Fig. 4, Supplementary Table S3). First, nitrate reductase allele 1 (*NIA1*) and NO synthase 1 (*NOA1*), that encodes for the main enzyme responsible for NO production (Gupta et al., 2011), were both repressed during Citrus non-host response to Xcv. On the other hand, the non-symbiotic hemoglobin 1 gene (*AHB1*), coding for a major NO-oxidizing enzyme (Mur et al., 2012), was strongly induced. An increase in NO content, including *AHB1* down-regulation, was reported during Arabidopsis host response to *P. syringae* pv. *tomato* and *Botrytis cinerea* (Mur et al., 2012). The NO diminution observed in our analysis, besides being part of a non-host response, could be a novel characteristic for Citrus or woody plants. Also, as NO blocks auxin signaling repressors degradation (Terrile et al., 2012), NO depletion agrees with the auxin down-regulation signaling mentioned before. In addition, this decrease of NO correlates with the diminution of cell death previously observed in Citrus response to Xcv (Daurelio et al., 2013), as cell death is associated to a NO increase (Gro et al., 2013).

3.5. PAs pathways show specific regulation patterns in Citrus non-host response

The levels of PAs suffer profound changes in plant tissues during the interaction with microorganisms (Jiménez-Bremont et al., 2014). Overall, these modifications are due to a coordinated regulation of PAs biosynthetic and catabolic pathways, and gene expression following microorganism recognition is intensely studied (Jiménez-Bremont et al., 2014).

In this work, Citrus PA pathways were analyzed during non-host response to Xcv. First, Citrus alleles of PA pathways were detected by a phylogenetic analysis comparing with those from *Arabidopsis* (Supplementary Fig. S2-S3). Arginine decarboxylase (*ADC*) and spermidine synthase (*SPDS*) presented one allele in comparison with the two alleles from *Arabidopsis* (Supplementary Fig. S2-S3). In contrast, polyamine oxidase (*PAO*), S-adenosylmethionine decarboxylase (*SAMDC*), spermine synthase (*SPMS*), thermospermine synthase (*ACL5*), N-carbamoylputrescine amidohydrolase (*NLP1*) and agmatine iminohydrolase (*AIH*) enzymes were identified in Citrus and presented a similar number

of alleles as those described in *Arabidopsis* (Supplementary Fig. S2-S3). The phylogenetic trees of biosynthesis and degradation genes from *C. sinensis* and *C. clemenules* were similar, supporting the alleles detected (Supplementary Fig. S2-S3).

All the PA pathways genes were represented in the microarray. Regarding those involved in biosynthesis, Arginine decarboxylase 1 (*ADC*), S-adenosylmethionine synthetases (*MAT2* and *MAT4*) were induced; while Spermidine synthase 1 (*SPDS1*), arginine deiminase (*AIH*), Nitrilase-like protein 1 (*NLP1*) and ornithine decarboxylase (*ODC*) were repressed in Citrus response to Xcv (Fig. 5A, Supplementary Table S3, Supplementary Fig. S4-S5). This PA biosynthetic pathway repression downstream to ADC, that includes the next enzyme AIH, could produce a deviation to agmatine, a precursor in the synthesis of hydroxycinnamic acid amides (HCAAs), secondary metabolites involved in plant defense against pathogens (Muroi et al., 2009). In addition, an overlap between PAs and NO responses during salinity stress has been postulated in Citrus plants (Tanou et al., 2014), being L-arginine the common precursor in both biosynthetic routes. So, the depletion of L-arginine by *ADC* induction agrees with the NO diminution postulated here for Citrus non-host response. The expression pattern of *ADC* was analyzed by real-time PCR, confirming the induction at 8 hpi observed in the microarray, then returning to baseline at 24 hpi (Fig. 5B). A similar *ADC* expression pattern was previously reported in tobacco non-host response to Xcc (Daurelio et al., 2011).

An alternative pathway for PAs biosynthesis is by means of arginase, which transcription level was constant in Citrus non-host response (data not shown). Arginase plays an important role in regulating the metabolism of L-arginine under stress (Chen et al., 2004). While in tomato, under biotic stress, this enzyme is induced through JA-regulation (Chen et al., 2004), in Citrus response to Xcv its levels remained unchanged, in accordance with the constant levels of JA observed. This result agrees with the weak arginase activity observed in a partial resistant to *Plasmidhiophora brassicae Arabidopsis* ecotype, while the susceptible one presented strong arginase activity (Jiménez-Bremont et al., 2014). Besides, *MAT2-4* induction and *SAMDC* repression lead to S-adenosyl-methionine, a precursor of ET biosynthesis, in agreement with the proposed increase in ET.

On the other hand, in the catabolic pathway, *PAO1* and *PAO4* were induced, while the copper-containing amine oxidase gene (*CuAO*) was repressed (Fig. 5A, Supplementary Table S3,

Supplementary Fig. S4-S5). The *PAO1* induction at 8 hpi observed in the microarray was confirmed by real-time PCR, then returning to baseline at 24 hpi (Fig. 5B). The PA catabolism by PAO1 and PAO4 could generate H_2O_2, as was observed during Citrus non-host response (Daurelio et al., 2013). Also, PA degradation by PAO leads to 1,3-diaminopropane, precursor for β-alanine, and to 4-aminobutanal, that conducts to γ-aminobutiric acid (GABA), both compounds involved in plant-pathogen response (Kim et al., 2013).

Finally, to elucidate the effect of transcriptional regulation of genes involved in Citrus PAs pathways on PAs content during non-host response, the free Put, Spm and Spd levels were quantified. At 8 hpi, only Put levels showed significant differences, with a diminution in Xcv and Xcc treatments compared to Ctr (Fig. 5C). Then, the levels of Put increased significantly at 24 hpi in Xcv response, and at 12 and 24 hpi in Xcc, in comparison to Ctr (Fig. 5C). On the other hand, Spd presented an increase at 12 and 24 hpi in Xcv and at 24 hpi in Xcc, comparing to Ctr (Fig. 5C). Finally, Spm quantification showed no significant variations (Fig. 5C).

It has been proposed that PAs mediate the activation of plant defense mechanisms (Jiménez-Bremont et al., 2014). In Citrus, this activation could involve a type of interaction-dependent regulation of free PAs levels, because the patterns observed in response to Xcv and Xcc were different. The diminution of Put at 8 hpi could be part of PTI basal response to both bacteria. In Citrus non-host response to Xcv, the rise in Spd at 12 hpi could be generated by Spm oxidation, due to PAO1 and PAO4 that catalyze the back conversion of Spm to Spd (Jiménez-Bremont et al., 2014). These enzymes also convert Spd in Put, and could cause the Put increase observed at 24 hpi (Supplementary Fig. S5). Even though a decrease in Spm should be expected, PA-conjugates (not measured here) could be replenishing Spm levels. The inverse fluctuation in Put and Spd levels in Citrus canker shows a specific fine tune in PAs levels that could conduct to defense or illness. Interestingly, the fact that PAs levels in Citrus canker are not regulated transcriptionally, because the enzymes of PAs pathways remain unchanged, indicates another type of regulation by Xcc that will be explored in future studies.

4. Conclusions

Altogether, the analysis of phytohormones participation during Citrus non-host response to Xcv showed that:

ABA content decreased, as predicted by the transcriptomic analysis.

SA participation can be suggested, given that SA gene markers and SA conjugating enzymes induction was observed, although SA levels remained invariable.

JA levels did not change, in agreement with the expression patterns observed for JA-related genes.

PAs presented specific response patterns: the repression of the majority of synthetic enzymes suggests a deviation in PA synthesis to other pathways, while the induction in catabolic enzymes could be depleting the conjugated pool to generate ROS.

An increase of ET and a decrease of auxins, BR, CK NO, and GA are denoted by the transcriptomic analysis.

Hormone participation during Citrus non-host response is summarized in Supplementary Fig. S6.

Acknowledgments

The authors would like to thank the International Union of Biochemistry and Molecular Biology - Wood-Whelan fellowship program for funding LDD's stay at IVIA and SP's stay at UJI. We are also grateful to Sebastián Graziati for the technical support with plant materials.

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica of Argentina (ANPCyT, grant number BID-PICT-201-0426) and from the Consejo Nacional de Investigaciones Científicas y Ténicas of Argentina (CONICET, PIP 2012-2014 grant number 11420110100394) to LDD, from the ANPCyT (grant number BID-PICT-2010-1762) to EGO, from the Ministerio de Economía y Competitividad (MINECO, grant number AGL2013-42038R) to VA and AG-C, from the Programa de Cooperación Argentina-España (ES/09/10, Ministerio de Ciencia, Tecnología e Innovación Productiva of Argentina, Ministerio de Ciencia e Innovación of España) to EGO, and from the Subprograma de Acciones Integradas - Ministerio de Ciencia e Innovación (AR2009-0023) of España to FRT. EGO and LDD are staff members of the CONICET (Argentina).

References

Adie, B.A., Perez-Perez, J., Perez-Perez, M.M., Godoy, M., Sanchez-Serrano, J.J., Schmelz, E.A., and Solano, R. (2007). ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. *Plant Cell* 19**,** 1665-1681. doi: 10.1105/tpc.106.048041.

Arbona, V., and Gómez-Cadenas, A. (2008). Hormonal modulation of *Citrus* responses to flooding. *J. Plant Growth Regul.* 27**,** 241-250. doi: 10.1007/s00344-008-9051-x.

Argueso, C.T., Ferreira, F.J., Epple, P., To, J.P.C., Hutchison, C.E., Schaller, G.E., Dangl, J.L., and Kieber, J.J. (2012). Two-component elements mediate interactions between cytokinin and salicylic acid in plant immunity. *PLoS Genetics* 8**,** e1002448. doi: 10.1371/journal.pgen.1002448.

Barbez, E., Kubes, M., Rolcik, J., Beziat, C., Pencik, A., Wang, B., Rosquete, M.R., Zhu, J., Dobrev, P.I., Lee, Y., Zazimalova, E., Petrasek, J., Geisler, M., Friml, J., and Kleine-Vehn, J. (2012). A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. *Nature* 485**,** 119-122. doi: 10.1038/nature11001.

Bari, R., and Jones, J.D. (2009). Role of plant hormones in plant defence responses. *Plant Mol. Biol.* 69**,** 473-488. doi: 10.1007/s11103-008-9435-0.

Brunings, A.M., and Gabriel, D.W. (2003). *Xanthomonas citri*: breaking the surface. *Mol. Plant Pathol.* 4**,** 141-157. doi: 10.1046/j.1364-3703.2003.00163.x.

Cao, F.Y., Yoshioka, K., and Desveaux, D. (2011). The roles of ABA in plant–pathogen interactions. *J. Plant Res.* 124**,** 489-499. doi: 10.1007/s10265-011-0409-y.

Chen, C.N., Chu, C.C., Zentella, R., Pan, S.M., and Ho, T.H. (2002). AtHVA22 gene family in Arabidopsis: phylogenetic relationship, ABA and stress regulation, and tissue-specific expression. *Plant Mol. Biol.* 49**,** 633-644.

Chen, H., McCaig, B.C., Melotto, M., He, S.Y., and Howe, G.A. (2004). Regulation of plant arginase by wounding, jasmonate, and the phytotoxin coronatine. *J. Biol. Chem.* 279**,** 45998-46007. doi: 10.1074/jbc.M407151200.

Chen, H., Xue, L., Chintamanani, S., Germain, H., Lin, H., Cui, H., Cai, R., Zuo, J., Tang, X., Li, X., Guo, H., and Zhou, J.M. (2009). Ethylene Insensitive3 and Ethylene Insensitive3-like1 repress salicylic acid Induction Deficient2 expression to negatively regulate plant innate immunity in *Arabidopsis*. *Plant Cell* 21**,** 2527-2540. doi: 10.1105/tpc.108.065193.

Chen, Z., Agnew, J.L., Cohen, J.D., He, P., Shan, L., Sheen, J., and Kunkel, B.N. (2007). *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. *Proc. Natl. Acad. Sci. U.S.A.* 104**,** 20131-20136. doi: 10.1073/pnas.0704901104.

Daurelio, L.D., Checa, S.K., Barrio, J.M., Ottado, J., and Orellano, E.G. (2009). Characterization of *Citrus sinensis* type 1 mitochondrial alternative oxidase and expression analysis in biotic stress. *Biosci. Rep.* 30**,** 59-71, 51. doi: 10.1042/BSR20080180.

Daurelio, L.D., Petrocelli, S., Blanco, F., Holuigue, L., Ottado, J., and Orellano, E.G. (2011). Transcriptome analysis reveals novel genes involved in nonhost response to bacterial infection in tobacco. *J Plant Physiol.* 168**,** 382-391. doi: 10.1016/j.jplph.2010.07.014.

Daurelio, L.D., Romero, M.S., Petrocelli, S., Merelo, P., Cortadi, A.A., Talon, M., Tadeo, F.R., and Orellano, E.G. (2013). Characterization of *Citrus sinensis* transcription factors closely associated with the non-host response to *Xanthomonas campestris* pv. *vesicatoria*. *J. Plant Physiol.* 170**,** 934-942. doi: 10.1016/j.jplph.2013.01.011.

Daurelio, L.D., Tondo, M.L., Romero, M.S., Merelo, P., Cortadi, A.A., Talon, M., Tadeo, F.R., and Orellano, E.G. (2015). Novel insights into the *Citrus sinensis* nonhost response suggest photosynthesis decline, abiotic stress networks and secondary metabolism modifications. *Funct. Plant Biol.* 42**,** 758-769. doi: 10.1071/FP14307.

Du, L., and Poovaiah, B.W. (2005). Ca^{2+}/calmodulin is critical for brassinosteroid biosynthesis and plant growth. *Nature* 437**,** 741-745. doi: 10.1038/nature03973.

Du, Z., Zhou, X., Ling, Y., Zhang, Z., and Su, Z. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic Acids Res.* 38**,** W64-W70. doi: 10.1093/nar/gkq310.

Durgbanshi, A., Arbona, V., Pozo, O., Miersch, O., Sancho, J.V., and Gómez-Cadenas, A. (2005). Simultaneous determination of multiple phytohormones in plant extracts by liquid

chromatography-electrospray tandem mass spectrometry. *J. Agric. Food Chem.* 53**,** 8437-8442. doi: 10.1021/jf050884b.

Eckert, C., Xu, W., Xiong, W., Lynch, S., Ungerer, J., Tao, L., Gill, R., Maness, P.-C., and Yu, J. (2014). Ethylene-forming enzyme and bioethylene production. *Biotechnol. Biofuels* 7**,** 1-11. doi: 10.1186/1754-6834-7-33.

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J.G. (2002). The *Arabidopsis* mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. *Plant Cell* 14**,** 1557-1566. doi: 10.1105/tpc.002022.

Fujita, Y., Fujita, M., Satoh, R., Maruyama, K., Parvez, M.M., Seki, M., Hiratsu, K., Ohme-Takagi, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2005). AREB1 is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in *Arabidopsis*. *Plant Cell* 17**,** 3470-3488. doi: 10.1105/tpc.105.035659.

Gro, F., Durner, J., and Gaupels, F. (2013). Nitric oxide, antioxidants and prooxidants in plant defence responses. *Front. Plant Sci.* 4:419. doi: 10.3389/fpls.2013.00419.

Gupta, K.J., Fernie, A.R., Kaiser, W.M., and van Dongen, J.T. (2011). On the origins of nitric oxide. *Trends Plant Sci.* 16**,** 160-168. doi: 10.1016/j.tplants.2010.11.007.

Hall, B.P., Shakeel, S.N., Amir, M., Haq, N.U., Qu, X., and Schaller, G.E. (2012). Histidine kinase activity of the ethylene receptor ETR1 facilitates the ethylene response in *Arabidopsis*. *Plant Physiol.* 159**,** 682-695. doi: 10.1104/pp.112.196790.

Hou, B., Lim, E.K., Higgins, G.S., and Bowles, D.J. (2004). N-Glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. *J. Biol. Chem.* 279**,** 47822-47832. doi: 10.1074/jbc.M409569200.

Hunter, K.J. (1998). A dansyl chloride-HPLC method for the determination of polyamines. *Methods Mol. Biol.* 79**,** 119-123. doi: 10.1385/0-89603-448-8:119.

Hutchison, C.E., Li, J., Argueso, C., Gonzalez, M., Lee, E., Lewis, M.W., Maxwell, B.B., Perdue, T.D., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2006). The *Arabidopsis* histidine phosphotransfer proteins are redundant positive regulators of cytokinin signaling. *Plant Cell* 18**,** 3073-3087. doi: 10.1105/tpc.106.045674.

556 Jiménez-Bremont, J.F., Marina, M., Guerrero-González, M.L., Rossi, F.R., Sánchez-Rangel, D.,
557 Rodríguez-Kessler, M., Ruiz, O.A., and Gárriz, A. (2014). Physiological and molecular
558 implications of plant polyamine metabolism during biotic interactions. *Front. Plant Sci.* 5:95.
559 doi: 10.3389/fpls.2014.00095.
560 Kim, N.H., Kim, B.S., and Hwang, B.K. (2013). Pepper arginine decarboxylase is required for
561 polyamine and γ-aminobutyric acid signaling in cell death and defense response. *Plant*
562 *Physiol.* 162**,** 2067-2083. doi: 10.1104/pp.113.217372.
563 Korasick, D.A., Enders, T.A., and Strader, L.C. (2013). Auxin biosynthesis and storage forms. *J. Exp.*
564 *Bot.* 64**,** 2541-2555. doi: 10.1093/jxb/ert080.
565 Marcos, R., Izquierdo, Y., Vellosillo, T., Kulasekaran, S., Cascón, T., Hamberg, M., and Castresana,
566 C. (2015). 9-Lipoxygenase-derived oxylipins activate brassinosteroid signaling to promote
567 cell wall-based defense and limit pathogen infection. *Plant Physiol.* 169**,** 2324-2334. doi:
568 10.1104/pp.15.00992.
569 Markakis, M.N., Boron, A.K., Van Loock, B., Saini, K., Cirera, S., Verbelen, J.P., and Vissenberg, K.
570 (2013). Characterization of a small auxin-Up RNA (SAUR)-like gene involved in *Arabidopsis*
571 *thaliana* development. *PLoS ONE* 8**,** e82596. doi: 10.1371/journal.pone.0082596.
572 Markakis, M.N., De Cnodder, T., Lewandowski, M., Simon, D., Boron, A., Balcerowicz, D., Doubbo,
573 T., Taconnat, L., Renou, J.P., Hofte, H., Verbelen, J.P., and Vissenberg, K. (2012).
574 Identification of genes involved in the ACC-mediated control of root cell elongation in
575 *Arabidopsis thaliana*. *BMC Plant Biol.* 12**,** 208. doi: 10.1186/1471-2229-12-208.
576 Mauri, N., Fernandez-Marcos, M., Costas, C., Desvoyes, B., Pichel, A., Caro, E., and Gutierrez, C.
577 (2016). GEM, a member of the GRAM domain family of proteins, is part of the ABA
578 signaling pathway. *Sci. Rep.* 6**,** 22660. doi: 10.1038/srep22660.
579 Mishina, T.E., and Zeier, J. (2007). Bacterial non-host resistance: interactions of Arabidopsis with
580 non-adapted Pseudomonas syringae strains. *Physiol. Plant.* 131**,** 448-461. doi: 10.1111/j.1399-
581 3054.2007.00977.x
582 PPL977 [pii].

583 Mur, L.A.J., Sivakumaran, A., Mandon, J., Cristescu, S.M., Harren, F.J.M., and Hebelstrup, K.H.
584 (2012). Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance
585 mechanisms against pathogens. *J. Exp. Bot.* 63**,** 4375-4387. doi: 10.1093/jxb/ers116.
586 Muroi, A., Ishihara, A., Tanaka, C., Ishizuka, A., Takabayashi, J., Miyoshi, H., and Nishioka, T.
587 (2009). Accumulation of hydroxycinnamic acid amides induced by pathogen infection and
588 identification of agmatine coumaroyltransferase in *Arabidopsis thaliana*. *Planta* 230**,** 517-527.
589 doi: 10.1007/s00425-009-0960-0.
590 Nakajima, M., Shimada, A., Takashi, Y., Kim, Y.C., Park, S.H., Ueguchi-Tanaka, M., Suzuki, H.,
591 Katoh, E., Iuchi, S., Kobayashi, M., Maeda, T., Matsuoka, M., and Yamaguchi, I. (2006).
592 Identification and characterization of Arabidopsis gibberellin receptors. *Plant J.* 46**,** 880-889.
593 doi: 10.1111/j.1365-313X.2006.02748.x.
594 Navarro, L., Bari, R., Achard, P., Lison, P., Nemri, A., Harberd, N.P., and Jones, J.D. (2008).
595 DELLAs control plant immune responses by modulating the balance of jasmonic acid and
596 salicylic acid signaling. *Curr. Biol.* 18**,** 650-655. doi: 10.1016/j.cub.2008.03.060.
597 Ndamukong, I., Abdallat, A.A., Thurow, C., Fode, B., Zander, M., Weigel, R., and Gatz, C. (2007).
598 SA-inducible *Arabidopsis* glutaredoxin interacts with TGA factors and suppresses JA-
599 responsive *PDF1.2* transcription. *Plant J.* 50**,** 128-139. doi: 10.1111/j.1365-
600 313X.2007.03039.x.
601 Parry, G., Calderon-Villalobos, L.I., Prigge, M., Peret, B., Dharmasiri, S., Itoh, H., Lechner, E., Gray,
602 W.M., Bennett, M., and Estelle, M. (2009). Complex regulation of the TIR1/AFB family of
603 auxin receptors. *Proc. Natl. Acad. Sci. U.S.A.* 106**,** 22540-22545. doi:
604 10.1073/pnas.0911967106.
605 Parry, G., Ward, S., Cernac, A., Dharmasiri, S., and Estelle, M. (2006). The *Arabidopsis* Suppressor of
606 Auxin Resistance proteins are nucleoporins with an important role in hormone signaling and
607 development. *Plant Cell* 18**,** 1590-1603. doi: 10.1105/tpc.106.041566.
608 Poppenberger, B., Fujioka, S., Soeno, K., George, G.L., Vaistij, F.E., Hiranuma, S., Seto, H.,
609 Takatsuto, S., Adam, G., Yoshida, S., and Bowles, D. (2005). The UGT73C5 of *Arabidopsis*

610 *thaliana* glucosylates brassinosteroids. *Proc. Natl. Acad. Sci. U.S.A.* 102**,** 15253-15258. doi:
611 10.1073/pnas.0504279102.
612 Priya, R., and Siva, R. (2015). Analysis of phylogenetic and functional diverge in plant nine-cis
613 epoxycarotenoid dioxygenase gene family. *J. Plant Res.* 128**,** 519-534. doi: 10.1007/s10265-
614 015-0726-7.
615 Rani, T.S., Durgeshwar, P., and Podile, A.R. (2015). Accumulation of transcription factors and cell
616 signaling-related proteins in the nucleus during citrus–Xanthomonas interaction. *J. Plant*
617 *Physiol.* 184**,** 20-27. doi: http://dx.doi.org/10.1016/j.jplph.2015.05.013.
618 Rani, T.S., and Podile, A.R. (2014). Extracellular matrix-associated proteome changes during non-host
619 resistance in citrus–Xanthomonas interactions. *Physiol. Plant.* 150**,** 565-579. doi:
620 10.1111/ppl.12109.
621 Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist
622 programmers. *Methods Mol. Biol.* 132**,** 365-386. doi: 10.1385/1-59259-192-2:365.
623 Senthil-Kumar, M., and Mysore, K.S. (2013). Nonhost resistance against bacterial pathogens:
624 retrospectives and prospects. *Annu. Rev. Phytopathol.* 51**,** 407-427. doi: doi:10.1146/annurev-
625 phyto-082712-102319.
626 Song, J.T. (2006). Induction of a salicylic acid glucosyltransferase, AtSGT1, is an early disease
627 response in *Arabidopsis thaliana*. *Mol. Cells* 22**,** 233-238.
628 Talon, M., and Gmitter, F.G., Jr. (2008). Citrus genomics. *Int. J. Plant Genomics* 2008**,** 528361. doi:
629 10.1155/2008/528361.
630 Tanou, G., Ziogas, V., Belghazi, M., Christou, A., Filippou, P., Job, D., Fotopoulos, V., and
631 Molassiotis, A. (2014). Polyamines reprogram oxidative and nitrosative status and the
632 proteome of citrus plants exposed to salinity stress. *Plant Cell Environ.* 37**,** 864-885. doi:
633 10.1111/pce.12204.
634 Terrile, M.C., Paris, R., Calderon-Villalobos, L.I., Iglesias, M.J., Lamattina, L., Estelle, M., and
635 Casalongue, C.A. (2012). Nitric oxide influences auxin signaling through S-nitrosylation of
636 the *Arabidopsis* Transport Inhibitor Response 1 auxin receptor. *Plant J.* 70**,** 492-500. doi:
637 10.1111/j.1365-313X.2011.04885.x.

638 Vanhee, C., Zapotoczny, G., Masquelier, D., Ghislain, M., and Batoko, H. (2011). The *Arabidopsis*
639 multistress regulator TSPO is a heme binding membrane protein and a potential scavenger of
640 porphyrins via an autophagy-dependent degradation mechanism. *Plant Cell* 23**,** 785-805. doi:
641 10.1105/tpc.110.081570.
642 Verma, V., Ravindran, P., and Kumar, P.P. (2016). Plant hormone-mediated regulation of stress
643 responses. *BMC Plant Biol.* 16**,** 86. doi: 10.1186/s12870-016-0771-y.
644 Wu, J., Wang, L., and Baldwin, I.T. (2008). Methyl jasmonate-elicited herbivore resistance: does
645 MeJA function as a signal without being hydrolyzed to JA? *Planta* 227**,** 1161-1168. doi:
646 10.1007/s00425-008-0690-8.
647 Yamauchi, Y., Takeda-Kamiya, N., Hanada, A., Ogawa, M., Kuwahara, A., Seo, M., Kamiya, Y., and
648 Yamaguchi, S. (2007). Contribution of gibberellin deactivation by AtGA2ox2 to the
649 suppression of germination of dark-imbibed *Arabidopsis thaliana* seeds. *Plant Cell Physiol.*
650 48**,** 555-561. doi: doi.org/10.1093/pcp/pcm023.
651 Youn, J.-H., Kim, M.K., Kim, E.-J., Son, S.-H., Lee, J.E., Jang, M.-S., Kim, T.-W., and Kim, S.-K.
652 (2016). ARF7 increases the endogenous contents of castasterone through suppression of *BAS1*
653 expression in *Arabidopsis thaliana*. *Phytochemistry* 122**,** 34-44. doi:
654 10.1016/j.phytochem.2015.11.006.
655 Zhang, X., Zhu, Z., An, F., Hao, D., Li, P., Song, J., Yi, C., and Guo, H. (2014). Jasmonate-activated
656 MYC2 represses Ethylene Insensitive3 activity to antagonize ethylene-promoted apical hook
657 formation in *Arabidopsis*. *Plant Cell* 26**,** 1105-1117. doi: 10.1105/tpc.113.122002.
658 Zhang, Y., Zhang, B., Yan, D., Dong, W., Yang, W., Li, Q., Zeng, L., Wang, J., Wang, L., Hicks,
659 L.M., and He, Z. (2011). Two *Arabidopsis* cytochrome P450 monooxygenases, CYP714A1
660 and CYP714A2, function redundantly in plant development through gibberellin deactivation.
661 *Plant J.* 67**,** 342-353. doi: 10.1111/j.1365-313X.2011.04596.x.
662 Zhiponova, M.K., Vanhoutte, I., Boudolf, V., Betti, C., Dhondt, S., Coppens, F., Mylle, E., Maes, S.,
663 González-García, M.P., Caño-Delgado, A.I., Inzé, D., Beemster, G.T.S., De Veylder, L., and
664 Russinova, E. (2013). Brassinosteroid production and signaling differentially control cell
665 division and expansion in the leaf. *New Phytol.* 197**,** 490-502. doi: 10.1111/nph.12036.

666

667

Figure Legends

Fig. 1. Citrus ABA-related genes differentially regulated during the non-host response to Xcv. (A-B) The log_2 of expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q<0.05$, using FDR correction). The A panel shows only genes related to ABA biosynthesis. (C) ABA levels in *C. sinensis* leaves inoculated with Xcv, Xcc and Ctr at 12 hpi. The averages of three repetitions with SE bars are represented. Asterisks indicate significant differences in the statistical analysis ($p<0.05$). "ns" indicate no significant differences in the statistical analysis.

Fig. 2. Citrus SA-related genes differentially regulated during the non-host response to Xcv. (A) The log_2 of expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q<0.05$, using FDR correction). (B) SA levels in *C. sinensis* leaves inoculated with Xcv, Xcc and Ctr at 12 hpi. The averages of three repetitions with SE bars are represented. "ns" indicate no significant differences in the statistical analysis.

Fig. 3. Citrus JA-related genes differentially regulated during the non-host response to Xcv. (A-B) The log_2 of expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q<0.05$, using FDR correction). (C) JA levels in *C. sinensis* leaves inoculated with Xcv, Xcc and Ctr at 12 hpi. The averages of three repetitions with SE bars are represented. Asterisks indicate significant differences in the statistical analysis ($p<0.05$). "ns" indicate no significant differences in the statistical analysis.

Fig. 4. Citrus hormone-related (ET, GA, auxins, BR, CK and NO) genes differentially regulated during the non-host response to Xcv. The log_2 of the expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q<0.05$, using FDR correction).

Fig. 5. Citrus PA-related genes differentially regulated during the non-host response to Xcv. (A) The log_2 of expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q<0.05$, using FDR correction). (B) Real-time PCR expression for ADC and PAO1 at 0, 8 and 24 hpi in the different systems. Both genes

were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons at 8 hpi ($p<0.05$). (C) Free PA content relative to the basal state. Graphs represent the subtractions of PA contents at each time from time 0 over the content in time 0 (Negatives values means that PA content decreased with respect to t=0; positive values means that PA content increased with respect to t=0). Significance is indicated by asterisk according to two-way ANOVA and Bonferroni post-test, where the difference is indicated in comparison to asterisk absence (* $p<0.05$).