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

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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.

Abbreviations: ABA, Abscisic Acid; *ABA1/ZEP*, Zeaxanthin epoxidase; *ABF3/DPBF5*, Abscisic acid responsive elements-binding factor 3; *ACL5*, thermospermine synthase; *ACO4*, Ethylene-forming enzyme/ACC oxidase; *ADC*, Arginine decarboxylase; *AHB1*, Nonsymbiotic Hemoglobin 1; *AHP5*, Histidine-containing Phosphotransferase protein 5; *AIH*, Agmatine iminohydrolase; ARF7, Auxin Response Factor 7; *ARGOS*, Auxin-Regulated Gene Involved in Organ Size; *BAS1*, PHYB Activation tagged Supressor 1; BR, Brassinosteroids; *BZR1*, Brassinosteroid signaling positive Regulator; *CCD4*, nine-cis-epoxycarotenoid dioxygenase 4; *CESA3*, Cellulose Synthase family protein; CK, Cytokinins; Ctr, Control; *CTR1*, Constitutive Triple Response 1; *CuAO*, Copper-containing amine oxidase gene; *CYP714A1*, Cytochrome P450 714A1; DEG, differentially expressed genes; *DIM*, Cell elongation protein DIMINUTO; *DOGT1*, DON-Glucosyltransferase 1; *DWF3*, Constitutive photomorphogenic DWARF; *EIN3*, Ethylene Insensitive 3 family protein; ET, Ethylene; ETI, Effector-triggered immunity; FDR, Hochberg false discovery rate; GA, Gibberellins; *GA2OX2*, Gibberellin 2-oxidase; *GAI*, Gibberellic Acid Insensitive; GEM, GLABRA2 expression modulator/GRAM domain protein; *GID1C*, Gibberellic acid Insensitive DWARF1C; *GRE5*, GEM-like protein 5/GRAM domain protein; *GRE8*, GEM-like protein 8/GRAM domain protein; *GRX480/GRXC9/GRX8*, Glutaredoxin 480; HCAAs, hydroxycinnamic acid amides; *HLS1/COP3*, *N*-acyltransferase Hookless 1/constitutive photomorphogenic 3; hpi, hours post infection; HR, Hypersensitive Response; *HVA22E*, Abscisic acid-induced protein homologue E/HVA22-like protein e; *ILL*, IAA amino acid conjugate hydrolase; JA, Jasmonic Acid or jasmonates; *JAZ1/TIFY10A*, Jasmonate-zim-domain protein 1; *KAT2/PED1*, Peroxisomal 3-ketoacyl-CoA thiolase 3; *MAP K3*, Mitogen-Activated Protein Kinase 3; *MAT*, *S*-adenosylmethionine synthetase; *MKK4*, Mitogen-activated protein Kinase Kinase 4; *MKK9*, Mitogen-Activated protein Kinase Kinase 9; *NIA1*, Nitrate Reductase 1; *NLP1*, Nitrilase-like protein 1; NO, Nitric Oxide; *NOA1*, Nitric Oxide Synthase 1; *ODC*, Ornithine decarboxylase; *PAD4*, Phytoalexin Deficient 4; PAMPs, Pathogen-associated molecular patterns; *PAO*, Polyamine oxidase; PAs, Polyamines; *PILS2*, Auxin efflux carrier family protein PIN-LIKES 2; *PR1*, Pathogenesis-Related protein 1; *PR5*, Pathogenesis-Related protein 5; PTI, PAMP-triggered immunity; Put, Putrescine; SA, Salicylic Acid; *SAMDC*, S-adenosylmethionine decarboxylase; *SAR1/NUP160*, Supressor of Auxin Resistance 1/Nuclear Pore complex protein NUP160; *SAUR*, Small Auxin up-regulated RNAs; SB, Silva Buddenhagen medium; SEA, Singular Enrichment Analysis; Spd, Spermidine; *SPDS*, Spermidine synthase; Spm, Spermine; *SPMS*, Spermine synthase; *TSPO*, Outer membrane Tryptophan-rich Sensory Protein-related; *UGT74F1*, UDP-glycosyltransferase 74F1; *UGT76C2*, UDP-Glucosyl Transferase 76C2; *VAS2/GH3.17*, Indole-3-acetic acid-amido synthetase GRETCHEN HAGEN 3.17; Xcc, *Xanthomonas citri* subsp. *citri*; Xcv, *Xanthomonas campestris* pv. *vesicatoria*

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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 Jr., 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 a 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 μE/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 28 °C 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).

2.3. Determination of plant hormone levels

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

2.4. Polyamines analysis

Control and treated leaves were harvested at 0, 8, 12 and 24 hpi, immediately frozen in liquid nitrogen, ground to a fine powder and lyophilized (−50 °C, 6 to 8 h). These times post infection were selected because the hormone levels changes should be posterior to the transcriptional modifications analyzed at 8 hpi, which was included in this case. Three biological replicates of leaves taken from three 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 4 °C 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 70 °C 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 × 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 −80 °C until RNA isolation. Total RNA was extracted using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol. RNA quality was tested using the OD_{260}/OD_{280} ratio and agarose gel electrophoresis. 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-PCR reactions were carried out in StepOne real-time PCR system (Applied Biosystems, USA) equipped with StepOne™ Software v2.2.2. Reactions were performed with 1 µl of cDNA template and a SYBR green-I reaction mixture containing 1:50,000 diluted SYBR green-I (Invitrogen), 10 pmol of each primer, 0.5 U Platinum-Taq DNA polymerase (Invitrogen), 40 mmol 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 was acquired during the 72 °C extension step. Specificity of the amplification reactions was assessed by melting curve analysis, which were run at 95 °C for 15 s and 60 °C for 15 s followed by an increase in temperature from 60 to 85 °C (0.2 °C/s) with continuous fluorescence recording. PCR reactions without the reverse transcription step did not yield products. In addition, real time PCR products using genomic DNA or cDNA templates for the actin housekeeping gene were sized differently, allowing the detection of genomic DNA contamination. To perform the analysis of relative expression, we used the $2^{-\Delta\Delta CT}$ method, where ΔCt represents the difference between Ct (cycle threshold) values of a target and the endogenous control (actin) in the same sample, and ΔΔCt is the difference between the ΔCt value of a particular sample and the mean of ΔCt of control samples used as reference (Daurelio et al., 2013).

2.6. Statistical and phylogenetic analysis

ABA, JA and SA hormone quantifications were statistically analyzed using a non-parametric Friedman test, considering plants as blocks, followed with ranks sum multiple comparisons. PAs quantifications were analyzed using a two-factor (treatment and time pi) mixed model ANOVA and DGC multiple comparison tests along with residual analysis and validation. Real time RT-PCR results were analyzed using One-way ANOVA and Bonferroni multiple comparison tests along with residual analysis and validation. Differences were considered statistically significant for p-values < 0.05.

Due to the complexity of PA pathways, *C. sinensis* and *C. clemenules* genes involved in the biosynthesis and degradation of PAs were identified by comparison with the sequences deposited in the database using Phytozome (www.phytozome.net). For ornithine decarboxylase (ODC), which is not present in *Arabidopsis*, sequences from *Oryza sativa* and *Glycine* max were used. Phylogenetic trees for PAs genes pathways (5000 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. 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. An increase of ET and a decrease of auxins, BR, CK NO, and GA are denoted by the transcriptomic analysis.

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 (Figs. 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 (Figs. 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 expressed, indicating ABA diminution during Citrus-Xcv interaction (Fig. 1A and B, Supplementary Table S3). First of all, two alleles of zeaxanthin epoxidase (*ABA1/ZEP*) and nine-cis-epoxycarotenoid dioxygenase 4 (*CCD4*), enzymes that catalyze the first and a key regulated step of ABA biosynthesis respectively, were down-regulated (Priya and Siva, 2015). In addition, the genes that encode ABA-induced proteins HVA22E and ABA responsive elements-binding factor (*ABF3/DPBF5*) were also repressed (Chen et al., 2002;Fujita et al., 2005). This strong decrease in ABA levels suggested by transcriptomic analysis was confirmed through direct quantification of the hormone in *C. sinensis* leaves during non-host response to Xcv (Fig. 1C).

Also, in correlation with ABA decrease, a strong repression of ABA-induced outer membrane tryptophan-rich sensory protein coding gene (*TSPO*) was observed (Vanhee et al., 2011). It has been postulated that TSPO binds porphyrins, allowing their degradation through an autophagy-dependent mechanism (Vanhee et al., 2011). This lower level of TSPO leaves porphyrins available to continue with the tetrapyrrole pathway deviation to the sirohaem synthesis branch, as was previously postulated to occur during *C. sinensis* non-host response (Daurelio et al., 2015).

Therefore, our results indicate a clear decrease in ABA as part of the non-host response triggered after challenging *C. sinensis* with Xcv. The observed induction of the genes encoding for some GRAM domain ABA-induced proteins (GEM, GRE5 and GRE8) could be due to their responses to other regulators (Mauri et al., 2016).

3.3. Citrus non-host response can be associated to SA-ET positive signaling and absence of JA participation

Different studies performed with plants exposed to pathogen infections have shown increased levels of SA, JA and ET, pointing out

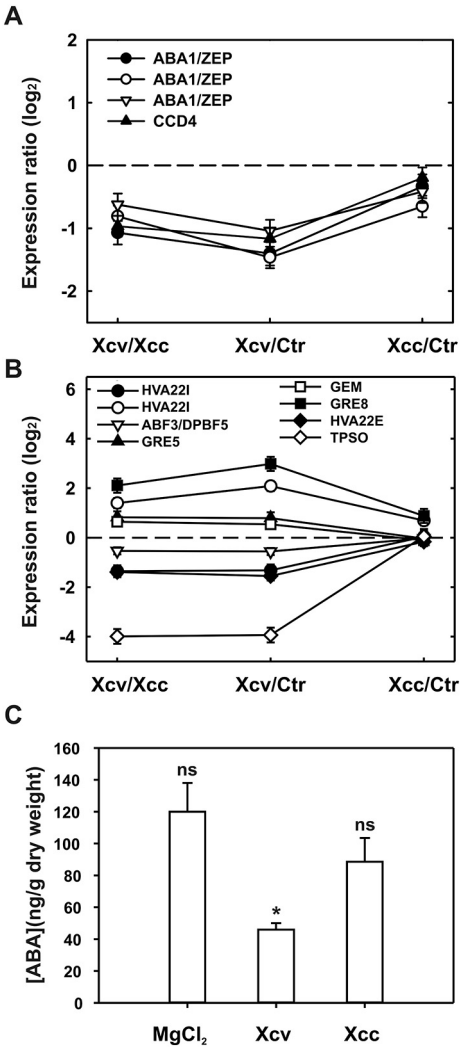

Fig. 1. Citrus ABA-related genes differentially regulated during the non-host response to Xcv. (A-B) The $\log_2$ of expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q < 0.05$, using FDR correction). The 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” indicates no significant differences in the statistical analysis.

these phytohormones as crucial elements in the regulation of plant defense against biotic stress. Particularly, SA seems to be responsible for defense against biotrophic and hemi-biotrophic pathogens, while ET and JA usually mediate the response to necrotrophic pathogens and herbivorous insects attack (Verma et al., 2016).

In our study, five up-regulated genes of the SA pathway were detected in Citrus non-host response (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

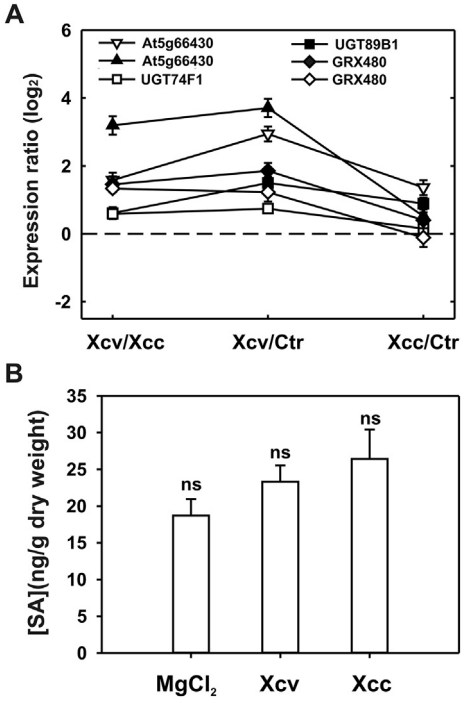

Fig. 2. Citrus SA-related genes differentially regulated during the non-host response to Xcv. (A) The $\log_2$ of expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q < 0.05$, using FDR correction). (B) SA 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" indicates no significant differences in the statistical analysis.

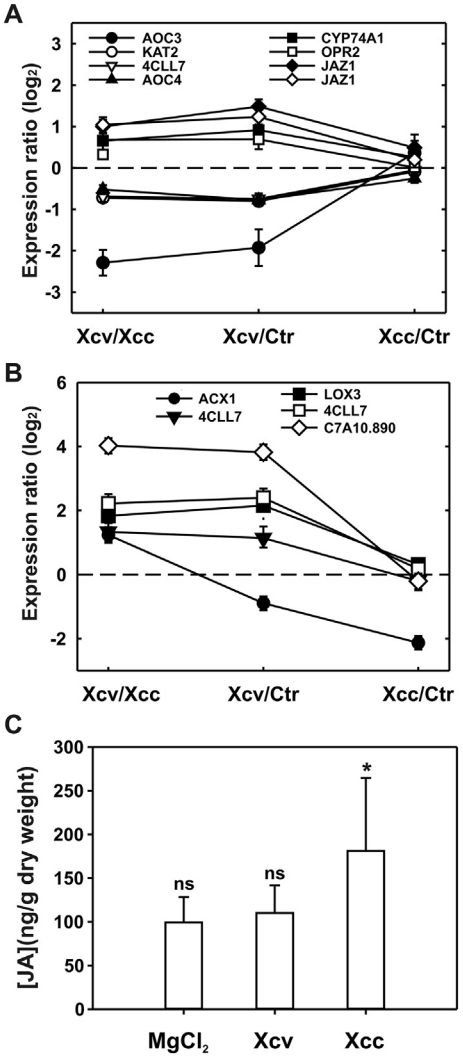

Fig. 3. Citrus JA-related genes differentially regulated during the non-host response to Xcv. (A-B) The $\log_2$ of expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q < 0.05$, using FDR correction). (C) 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" indicates no significant differences in the statistical analysis.

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

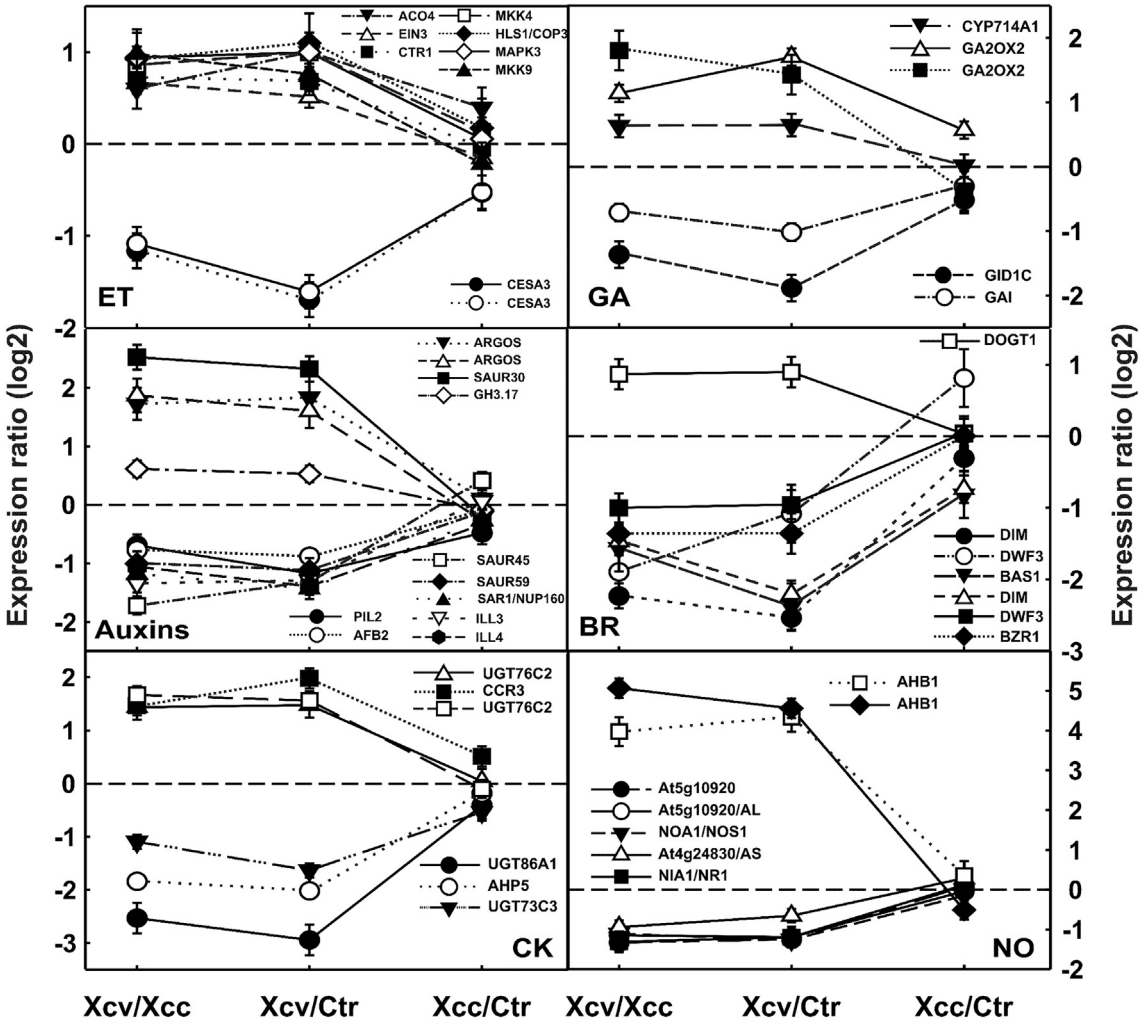

Fig. 4. Citrus hormone-related (ET, GA, auxins, BR, CK and NO) genes differentially regulated during the non-host response to Xcv. The $\log_2$ of the expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q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

(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 levels 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 BR 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, BR 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

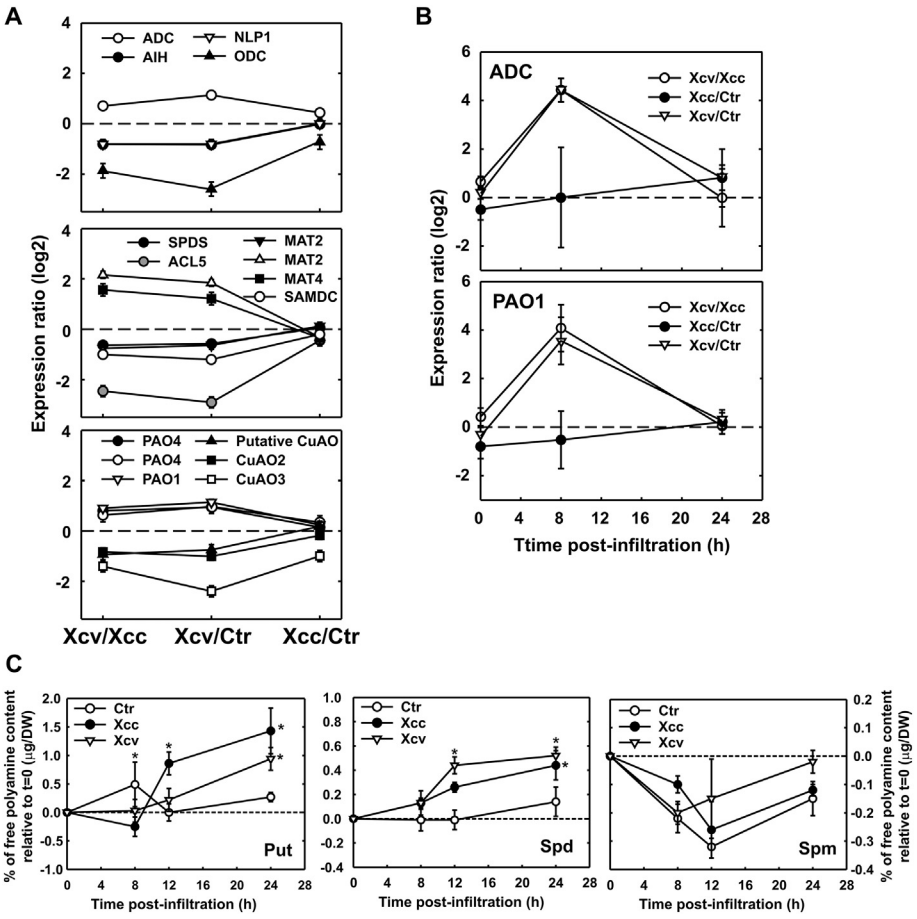

Fig. 5. Citrus PA-related genes differentially regulated during the non-host response to Xcv. (A) The $\log_2$ of expression ratio between treatments (M) with SE bars are shown. All these genes were statistically significant in Xcv-Xcc and Xcv-Ctr comparisons ($q < 0.05$, using FDR correction). (B) Real-time 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 (*$p < .05$).

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 nonsymbiotic 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).

Also, 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 Figs. S2–S3). Arginine decarboxylase (*ADC*) and spermidine synthase (*SPDS*) presented one allele in comparison with the two alleles from *Arabidopsis* (Supplementary Figs. 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 Figs. S2–S3). The phylogenetic trees of biosynthesis and degradation genes from *C. sinensis* and *C. clemenules* were similar, supporting the alleles detected (Supplementary Figs. 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 Figs. 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-adenosylmethionine, 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 Figs. 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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2018.05.006.

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