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Genetic analysis of salicylic acid-mediated defenses responses and histopathology in the *huanglongbing* pathosystem

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SUMMARY

The emergence of citrus *huanglongbing* (HLB) has been a constraint for worldwide citrus growers. HLB disease is associated to species of the biothrophic bacteria *Candidatus* Liberibacter spp. (CaL). In this study, we assessed the transcriptional status of salicylic acid (SA) genes and associated defenses between two contrasting citrus genotypes during challenge with *Ca*. Liberibacter asiaticus or *Ca*. Liberibacter americanus. *Citrus sinensis* exhibited the most evident alterations in gene expression of evaluated genes, when compared with *Poncirus trifoliata.* Upstream pathway SA genes showed a slight upward regulation in *C. sinensis*. Salicylic acid biosynthesis and accumulation might be impaired as we observed a low expression level of SA biosynthesis related genes. Moreover, genes associated to SA metabolism showed a slight induction. These results may account for the absence of significant downstream defense response related to salicylic acid. Leaf anatomical analysis revealed callose accumulation in both HLB infected, *C. sinensis* and *P. trifoliata* sieve tube elements (STE), although only *C. sinensis* exhibited collapsed STE. Our data corroborate other studies and suggest that the SA biosynthesis and metabolism related genes might be involved in the contrasting response to CaL in different citrus genotypes. Additionally, we suggest that collapsed STE might have a prominent implication in symptomatology of highly susceptible plants.

Index terms: SAR, plant-pathogen interaction, gene expression, callose deposition.

Análises genéticas das respostas de defesas mediadas por ácido salicílico e histopatologia no patossistema *huanglongbing*

RESUMO

A ocorrência do *huanglongbing* (HLB) dos citros tem sido um sério problema para os citricultores em todo o mundo. O HLB está associado a bactérias biotróficas da espécie *Candidatus* Liberibacter spp. (CaL). Nesse estudo, nós avaliamos o perfil transcricional dos genes da via do ácido salicílico (SA) e genes associados a defesa em dois genótipos contrastantes após desafio com *Ca*. Liberibacter asiaticus ou *Ca*. Liberibacter americanus. *Citrus sinensis* exibiu maiores alterações na expressão gênica dos genes avaliados, quando comparado com *Poncirus trifoliata.* Genes *upstream* da via do

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SA demonstraram aumento da expressão em *C. sinensis*. A biossíntese e acumulação do ácido salicílico deve ter sido prejudicada, uma vez que foi observado uma baixa expressão relativa dos genes relacionados a biossíntese do SA. Além disso, os genes associados ao metabolismo do SA tiveram uma leve indução da expressão. Esses resultados podem explicar a ausência de significância nos valores de expressão gênica dos genes *downstream* da via do SA, os quais são relacionados às respostas de defesa da planta. Análises anatômicas das folhas revelaram um acúmulo de calose nos elementos de tubo crivado (STE), tanto em *C. sinensis* quanto em *P. trifoliata* infectados. Nossos dados corroboram outros estudos e sugerem que os genes relacionados a biossíntese e metabolismo do SA podem estar envolvidos na resposta de genótipos de *Citrus* contrastantes à infecção por CaL. Além do mais, nós sugerimos que STE colapsados podem ter uma implicação importante na sintomatologia de plantas altamente suscetíveis.

Termos de indexação: SAR, interação planta-patógeno, expressão gênica, deposição de calose.

INTRODUCTION

Huanglongbing (HLB) is a systemic citrus disease associated with the bacteria *Candidatus* Liberibacter (CaL) (Wang et al., 2017; Wang & Trivedi, 2013). *Ca*. L. asiaticus (CLas) and *Ca*. L. americanus (CLam) are found in Brazil (Teixeira et al., 2005; Coletta-Filho et al., 2004) and the disease has caused significant economic impact to citrus growers (Bassanezi et al., 2011). Citrus typical symptoms are blotchy mottle; yellowed veins, leaves and shoots; root system decline; off-season flowering; small and lopsided fruits and dark aborted seeds (Gottwald, 2010; Bové, 2006). All of these symptoms occur because of multiple factors, including the callose accumulation in phloem sieve tube elements (STE) (Boava et al., 2017; Koh et al., 2012; Graça et al., 2016).

Callose deposition around the site of infection is considered a defense mechanism against pathogen colonization in host plants (Luna et al., 2011). In the HLB pathosystem, it has been considered the predominant factor associated with phloem plugging in CLas infected citrus, contributing to the progress of the disease (Wang et al., 2017; Koh et al., 2012). Callose may negatively regulate salicylic acid (SA) mediated defenses; on the other hand, the hormone SA may induce callose accumulation (Ellinger & Voigt, 2014; Nishimura, 2008). SA acts as a key regulator of plant defenses against a broad-spectrum group of pathogens (Tanaka et al., 2015). The increase of reactive species of oxygen (ROS) during the pathogen attack stimulates the SA biosynthesis and signaling (Pitino et al., 2017; Xing et al., 2013). Although SA and ROS levels increase systemically, sometimes they are not high enough to lead to a massive programmed cell death, so expression of defense related genes is activated instead (Kawano & Bouteau, 2013). This response is known as Systemic Acquired Resistance (SAR), an induced response that promotes resistance primarily against biotrophic pathogens (Ádám et al., 2018).

Several studies on citrus x CaL interaction have been done in pursuit of strategies to control the disease. However, HLB remains a challenge to citrus breeders and growers. We assessed the transcriptional profiles of SA-related SAR genes and performed a histopathological analysis of *C. sinensis* and *P. trifoliata* plants manifesting contrasting symptoms during infection, to better understand the plant response in this pathosystem.

MATERIALS AND METHODS

Plant material and experimental set up

Poncirus trifoliata (L.) Raf. plants were used as HLB tolerant/resistant genotype and *Citrus sinensis* (L.) Osbeck var. Hamlin as susceptible. For bacterial inoculation, plants were grafted with buds obtained from infected citrus plants. Non-grafted *P. trifoliata* and *C. sinensis* were used as control samples. Plants were maintained in greenhouse at 25±3°C under natural photoperiod. Two independent experiments were carried out using CLam or CLas. For gene expression analyses, time-course experiments of early [4 weeks after inoculation (wai)] and late stage of infection were performed. Late stage of infection was established as the moment when the inoculated plants exhibited typical HLB symptoms and ranged from 40 wai for CLas to 110 wai to CLam infected plants (Figure 1). Mature leaf samples were collected randomly, frozen into liquid nitrogen, grinded to a fine powder and kept at -80°C.

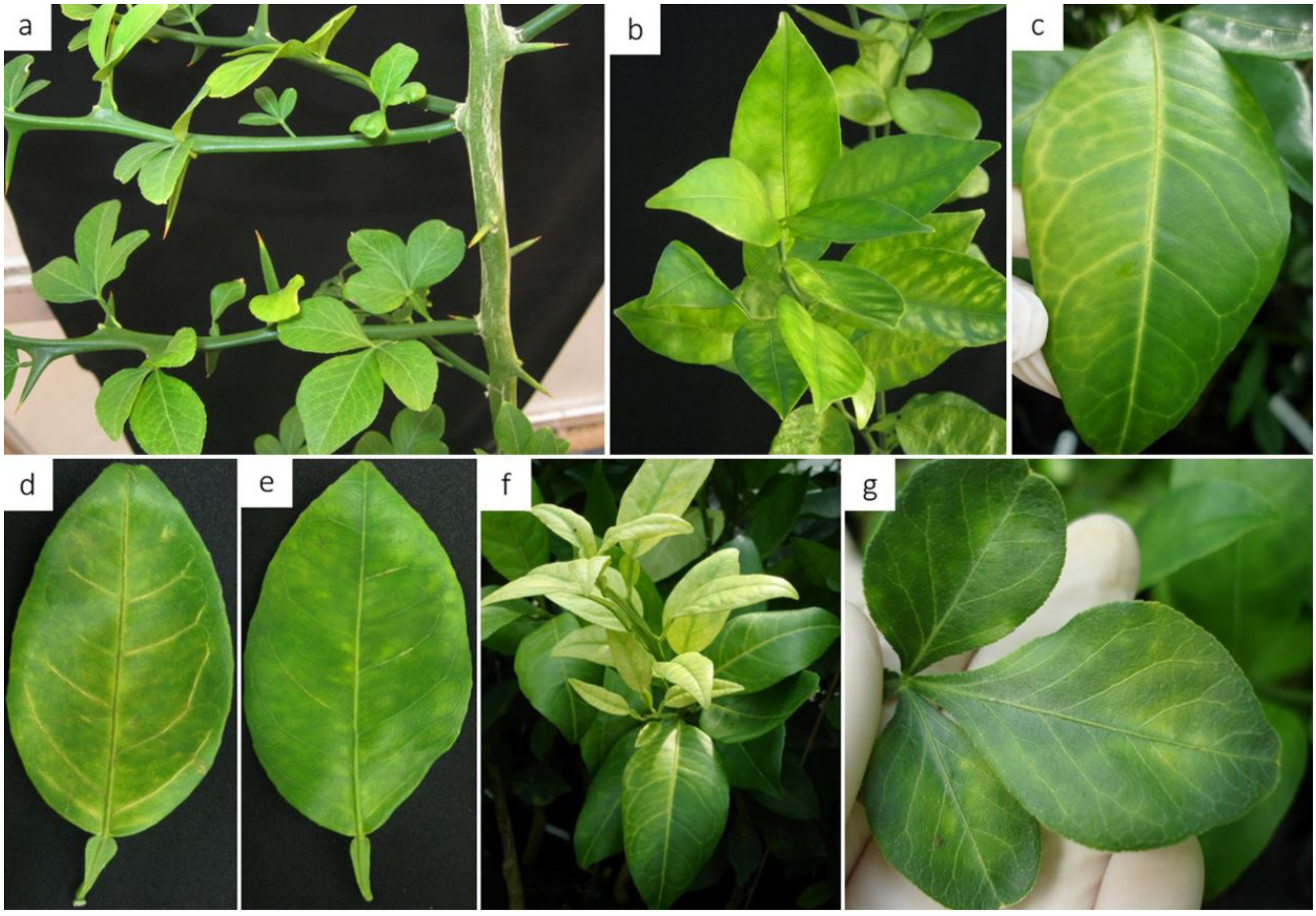

Figure 1. Plants of *Citrus sinensis* (b-f) and *Poncirus trifoliata* (a and g) inoculated with CLas (a, b, d, e) and CLam (c, f, g). Blotchy mottle leaves and shoots (b); leaves with veins corks and yellow sectors (d, e); yellowed veins (c, d); leaves without defined symptoms (a, g).

RNA isolation, cDNA synthesis and gene expression analysis

Total leaves RNA from CLas and CLam positive and healthy plants were extracted using the RNeasy Plant mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instruction. DNase I (QIAGEN) treatment was applied to degrade genomic DNA contaminants in RNA samples. The RNA concentration was determined by measuring the absorbance at 260 nm (A260) in a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA), and RNA integrity assessed by electrophoresis on 1.2% denaturing agarose gel. The ratio of OD at 260 and 280 nm was used to estimate the RNA purity. cDNA synthesis was carried using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada), from 1 µg of RNA and oligo(dT) primers, in a 20 µL reaction volume. For gene expression analysis, aliquots (3 µL) of 50-fold diluted cDNA samples were used as template in a 25 µL reaction containing 1 X Fast SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) and 200 nM of each specific primer. The reactions were carried out in an ABI PRISM 7500 FAST sequence detection system (Applied Biosystems) with a program of 95ºC for 20 seconds followed by 40 cycles of 95°C for 03 seconds and 60°C for 30 seconds.

Selection of salicylic acid-related SAR genes

Arabidopsis thaliana genes related to SA pathway were obtained on TAIR (Berardini et al., 2015) and used for a search of citrus orthologous genes by BLASTX on Phytozome (Goodstein et al., 2012). All these analyses were performed using BLAST default parameters. Real-time

quantitative PCR (qPCR) primers were designed using Primer3Plus software tool (Untergasser et al., 2007). For those genes belonging to gene families, qPCR primers were carefully obtained by observing an alignment of coding sequences and checking a unique annealing site between them.

The genes analyzed were grouped in categories according to their role along the SA pathway: 1) upstream pathway signaling; 2) SA biosynthesis and metabolism; 3) downstream pathway signaling. An Applied Biosystems 3730 capillary DNA sequencer (Applied Biosystems) was used to obtain the amplicon sequences, which were evaluated by aligning to the coding target sequence. Primers specificity was also verified by 2% (w/v) agarose gel electrophoresis of the PCR products and by qPCR melting curves. The description of the selected genes and primers sequences are summarized in Table 1.

Table 1. Citrus genes identification of salicylic acid (SA) pathway signaling. The analyzed genes were grouped in categories according to their role throughout salicylic acid pathway: upstream pathway signaling, SA biosynthesis and metabolism and downstream pathway signaling

AtID	PhytozomeID	Gene	Gene description	Primers (5'-3')
Upstream pathway signaling genes				
AT3G48090	orange1.1g007278m	EDS1	Component of R gene-mediated disease resistance	F TTTACAGGGCATTCCTCAGCTGGT R TATAGGTGGCATTCTGCTGGTGCT
AT3G52430	orange1.1g007061m	PAD4	Encodes a lipase-like protein that are important for SA-mediated defenses	F GCTTGGCAAATTCTGGGATGGTGT R ACCTCCTTGGTCTTCCATGCTTCA
AT4G11260	orange1.1g017264m	HSP90	Heat shock protein	F GCAAGCCATTGAGATTAGCC R GCCACCTTAGCCGTTTCATA
AT5G51700	orange1.1g027086m	RAR1	Required for mla12 resistance	F GCTCCTGCTCCTTCAACAAC R CTGGGGCATAATTGGTCTGT
AT5G06320	orange1.1g027210m	NDR1	Non-race specific disease resistance gene	F TTGTAACGCATGGGCTGAAACAG R TGGTGCAGGTCCTCAATCTTTGGA
AT1G73805	orange1.1g011961m	SARD1	Key regulator for ICS1 induction and SA synthesis	F GCCGAAGGAGTTTTGAATGA R AATTTGCCAATCGTTGCTTC
SA biosynthesis and metabolism related genes				
AT1G74710	orange1.1g008131m	ICS1	Isochorismate synthase	F TGCTCGATTGGCGGGCAGAC R AGGCGTGCCTCTTCAGTCGGA
AT3G53260	orange1.1g005031m	PAL2	Phenylalanine ammonia lyase	F GGCTGCCATTGCCACGTCCT R AGAGCACCGCCGTTCTTGGT
AT5G04230	orange1.1g004955m	PAL3	Phenylalanine ammonia lyase	F AACTGGACCGTGGCAGCGGA R GACTCGCCGCCGAGCTTCAC
AT3G10340	orange1.1g037382m	PAL4	Phenylalanine ammonia lyase	F TCACCGGCCGGCCCAATTCT R GCGAGACCCTCCTTAGGCTGC
-	orange1.1g016039m	PAL5	Phenylalanine ammonia lyase	F CTGACGATGGGGGTCAATGGGGA R CGCTGCAGGGATCATCAGCGTA
AT2G43820	orange1.1g012194m	SAGT1	Salicylic acid glucosyltransferase	F CCAAGCGCCTAGATCACAA R GGACGAGGATGACGAATCTC
AT3G11480	orange1.1g017514m	BSMT1	Benzoic acid/ SA carboxyl Methyltransferase	F GTTTAACGAGGCCGTTGATG R TCGTCAAGGAAACTGTCACG

Table 1. Continued...

AtID	PhytozomeID	Gene	Gene description	Primers (5'-3')
Downstream pathway signaling genes				
AT1G64280	orange1.1g007923m	NPR1	Nonexpresser of PR genes 1	F AAGGGAGCTCGGCCATCAGA R TGCAGCCTTAGTGAGCCGCT
AT5G45110	orange1.1g007849m	NPR3	Regulate defense responses against bacterial pathogens	F TCTGGAGGGAGAAATGAGG R TGTGGGAGGTGATAAAGGC
AT3G56400	orange1.1g021598m	WRKY70	WRKY70 transcription factor	F CAGCAGCAGCAGGCGAATTCTT R GTCCTTCGCCGCCGGTCTCT
AT4G03550	orange1.1g000259m	GSL5	Callose synthase	F CCCAAGTATTCCGGCCCTTT R CACGGGGGTCAAGACAATCA
AT4G04970	orange1.1g000258m	GSL1	Callose synthase	F CAGGTGTATGGGCAGCAGAA R CATCCCTCCCCAAGTGAACC

Data analysis

Efficiency of qPCR amplification and cycle threshold (Ct) were obtained using the Miner web-based tool from raw data of the kinetics of individual qPCR assay, according to Zhao & Fernald (2005). The relative quantification of gene expression level was calculated using the GenEx v2.6.4 software (MultiD analysis, Exiqon) and three biological and technical replicates were used for all conditions. We considered a ≤ 0.05 *p*-value for significant gene expression of ±1 - fold change, in a two-tailed t-test. The housekeeping genes *TIP41* and *SAND* were used as reference genes based on previous selection according to Mafra et al. (2012).

Microscopical analyses

For general anatomical analyses, petiole samples from CLas infected *P. trifoliata* and *C. sinensis* were collected and immediately fixed in Karnovsky solution (Karnovsky, 1965). Samples were submitted to a vacuum pump to remove the air in the intercellular spaces. After fixation, they were dehydrated in a graded series of ethyl alcohol and embedded in Leica Historesin® (Heraeus Kulzer, Hanau, Germany) media. Cross and longitudinal sections of 7-5 µm thick were cut with a rotatory microtome and stained with toluidine blue (Sakai, 1973) and the samples were mounted using synthetic resin Entelan®.

To study callose deposition, petioles were harvested, rapidly fixed in cold FAA fixative (95% ethanol, 37% formaldehyde, glacial acetic acid, deionized water, in the proportions of 10:2:1:7) and submitted to vacuum pump for 15 minutes. Longitudinal sections were made on sliding microtome (Leica MS 2000R), stained with 1% aniline blue (diluted in alcohol 70%) for 10 min and examined using a Filter A4 (excitation 340-380 nm, emission 450-490 nm) under Leica DMLB epifluorescence microscopy (Leica™ - Wetzlar, Germany) All digital images were acquired with a digital camera system with IM50 (Leica™ - Wetzlar, Germany) software.

RESULTS AND DISCUSSION

Here we provide an overview of the transcriptional profile for key SA-related SAR genes in contrasting genotypes upon *Ca*. Liberibacter spp. infection. To evaluate the dynamics of gene expression, the presence of both bacteria was confirmed in the infected plants (data not shown) and total RNA was isolated from leaf tissues collected in early (4 wai) and late (symptomatic) stages of infection, besides from healthy plants (non-inoculated). CLas-infected *P. trifoliata* plants lacked clear symptoms (Figure 1a); in contrast, CLas infected *C. sinensis* plants exhibited typical symptoms 40 wai (Figure 1b, c, d, e), in agreement with the literature (Folimonova et al., 2009). As for CLam-infected *C. sinensis*, typical disease symptoms appeared around 110 wai (Figure 1f), while CLam infected *P. trifoliata* also remained asymptomatic (Figure 1g).

In general, when comparing inoculated and non-inoculated plants, the most significant difference in expression was observed to SA biosynthesis and metabolism associated genes (Table 2; Figure 2). Almost all SA biosynthesis related genes showed a downward trend in expression, including

Table 2. Comparison of the expression levels of salicylic acid pathway-related SAR genes in *Poncirus trifoliata* and *Citrus sinensis* upon *Candidatus* Liberibacter americanus (CLam) or *Ca*. Liberibacter asiaticus (CLas) infection. The analyses were performed in early (ES) and late stages (LS) of infection. The analyzed genes were grouped in categories according to their role throughout salicylic acid pathway: upstream pathway signaling, SA biosynthesis and metabolism, and downstream pathway signaling

Genes	Trifoliata/CLam		Trifoliata/CLas		Citrus/CLam		Citrus/CLas	
	Upstream pathway signaling genes							
	ES	LS	ES	LS	ES	LS	ES	LS
EDS1	-0.11	-0.20	-0.37	0.22	-0.63	0.90	0.13	2.64*
PAD4	-0.42	-0.29	0.98	0.66	-0.75	1.65*	-0.61	1.72*
HSP90	-0.73	0.67	-1.95*	1.59	-1.20	-1.60	-0.78	0.18
RAR1	0.30	0.61	0.36	0.44	0.10	-0.44	-1.10	0.33
NDR1	0.33	1.6*	-0.14	0.84	0.11	1.4*	-0.31	4.4*
SARD1	1.03	4.0*	0.74	0.76	0.17	0.62	0.04	-0.29
	SA biosynthesis and metabolism related genes							
	ES	LS	ES	LS	ES	LS	ES	LS
ICS1	-0.66*	-2.07*	0.31	-4.88*	1.24	-0.13*	-0.02	-0.33
PAL2	4.03*	-2.20	0.17	2.02	-0.41	-1.76*	-0.09	-2.42*
PAL3	2.44*	0.23	0.38	1.64	-1.88*	-0.83	-0.55	-2.56
PAL4	1.3*	-0.94	0.16	2.54	0.42	-1.97*	0.14	-2.69
PAL5	1.85	-1.51	1.07	3.14	3.44	-2.22*	0.48	-5.62
SAGT1	-1.0*	0.47	-0.32	0.87	-0.56	1.90*	-0.33	1.30
BSMT1	-0.26	0.8	-0.93	0.48	-0.22	2.65*	-2.62	3.71*
	Downstream pathway signaling genes							
	ES	LS	ES	LS	ES	LS	ES	LS
NPR1	0.08	-0.33	-0.23	-1.2*	-0.15	-0.09*	0.08	-0.03
NPR3	2.86*	-0.50	0.26	0.08	0.16	0.92	-0.27	-1.18*
WRKY70	-0.44*	0.33	-0.31	1.64	0.55	0.27	-0.11	0.10
GSL5	-1.06	-0.47	0.03	-0.10	1.78	-1.67*	-0.26	0.37*
GSL1	0.58	-0.07	-1.16	0.02	1.16	-1.93*	-0.13	0.23

The differentially gene expression analyses were based on the fold ratio ≥1 or ≤-1 between infected and non-infected plants. Genes with a p-value ≤ 0.05 were considered significant and are present with asterisk.

ICS1 (*ISOCHORISMATE SYNTHASE1*) (Table 2, Figure 2). The isochorismate pathway produces 90% of plant SA (Wildermuth et al., 2001), while PAL (*PHENYLALANINE AMMONIA-LYASE*) pathway is responsible for the remaining SA production (Chen et al., 2009). PAL gene family also showed decrease of expression in late stages of infection, even though trifoliata exhibited increase of expression in early stage of infection (Figure 2). Slisz et al. (2012) showed phenylalanine accumulation on symptomatic citrus fruits infected with CLas, which could be due to inhibition of the phenylpropanoid biosynthetic pathway by the bacteria. Phenylpropanoids are induced in response to biotic and abiotic stresses and the initial step in the phenylpropanoid biosynthetic pathway is the conversion of phenylalanine to cinnamic acid by the action of PAL (Chen et al., 2009). Downregulation of SA biosynthesis genes can be explained by two hypotheses: SA level in both stages evaluated in this work is high, thus the expression of *ICS1* and *PAL* genes is low because SA synthesis is no more necessary or *ICS1* is potentially targeted by CaL. to suppress SA-mediated plant immunity, as showed to other plant pathogens (Qi et al., 2018).

For SA metabolism, *BSMT1* (*BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE1*) – a SA metabolic enzyme gene – had significant level of induction in late stage of infection of both CLas and CLam (Figure 2). This gene encodes a SABATH methyltransferase that catalyzes the methylation of SA into methylsalicylate (MeSA) and benzoic acid (a SA precursor) into methylbenzoate (MeBA) (Chen et al.,

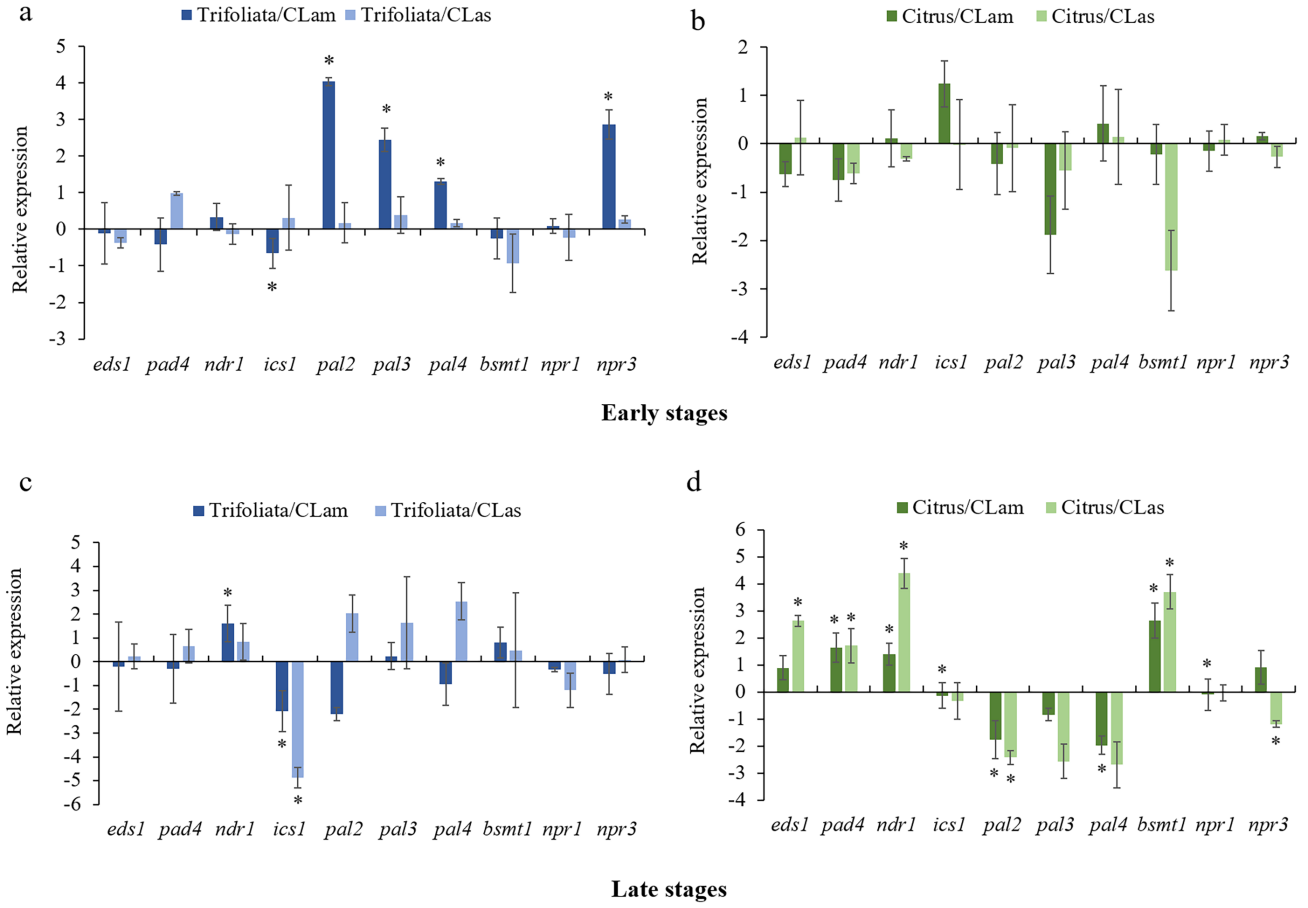

Figure 2. Relative expression of upstream SA pathway signaling genes and SA metabolism related genes in *Poncirus trifoliata* and *Citrus sinensis* upon *Candidatus* Liberibacter americanus (CLam) or *Ca*. Liberibacter asiaticus (CLas) infection. a. Graphic of relative expression of *P. trifoliata* in early stages of CLam and CLas infection. b. Graphic of relative expression of *C. sinensis* in early stages of CLam and CLas infection. c. Graphic of relative expression of *P. trifoliata* in late stages of CLam and CLas infection. d. Graphic of relative expression of *C. sinensis* in late stages of CLam and CLas infection. Genes with *p*-value ≤ 0.05 were considered significant and are present with asterisk.

2003). *BSMT1* mutants showed compromised SAR signaling, suggesting MeSA as a critical phloem-mobile SAR signal (Koo et al., 2007; Liu et al., 2010). However, MeSA seems not to be the only molecule essential to establish SAR (Attaran et al., 2009; Zheng et al., 2012; Maruri-López et al., 2019), and several candidate molecules have been suggested (Shah & Zeier, 2013). Moreover, some pathogens manipulate phytohormone pathways, converting SA to inactive derivatives as MeSA, to bypass defenses responses (Cui et al., 2005; Qi et al., 2018). In *A. thaliana*, *Pseudomonas syringae* prevents the SA accumulation by inducing the SA metabolic gene *BSMT1* and repressing SA synthesis gene *ICS1*, similar to what was observed in late stages of CLam inoculated-citrus plants (Table 2). Therefore, SA is converted to the volatile form (MeSA) and defenses against the pathogen are suppressed (Zheng et al., 2012). Interestingly, it has been shown that MeSA and other specific compounds are released by CLas-infected plants, which may render to citrus CLas hosts more attractiveness to the vector *Diaphorina citri* than non-infected plants (Mann et al., 2012). MeSA acts as an olfactory signal to *D. citri* (Grafton-Cardwell et al., 2013), but once the insects settle on infected leaves and possibly acquire the bacteria, they soon move to a healthy plant (Mann et al., 2012).

For upstream pathway signaling genes, *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*) and *NDR1/HIN1-like* (NHL) were the most up regulated in citrus after CLas infection (late stage) (Table 2; Figure 2). *EDS1* is an important regulator of SA-dependent defense response against biotic stress, for example, involved in hypersensitive response (HR), accumulation of

SA and SA-dependent signaling (Shah & Zeier, 2013; Rietz, et al. 2011). *NDR1/HIN1-like* is a plasma membrane protein, member of a gene family involved in multiple roles in cell physiology (Knepper et al., 2011). Previous studies have identified increase in expression of *NDR1/HIN1-like* related genes during CLas or CLam infection in *C. sinensis* (Lu et al., 2013; Mafra et al., 2013). Vilaine et al. (2013) showed that arabidopsis overexpressing *NHL26* accumulated high levels of carbohydrate in phloem tissue of mature leaves, higher shoot biomass, contrasting with slower root growth and a lower seed yield. HLB infected citrus species manifest similar characteristics, which have been considered a consequence of phloem blockage (Johnson et al., 2014; Wang & Trivedi, 2013). Besides that, PAD4 (*PHYTOALEXIN DEFICIENT4*) is a lipase-like protein which showed a slight up regulation in CLas infected citrus in the late stage of infection (Table 2). Overexpression of EDS1 and PAD4 activates the expression of both SA-dependent and SA-independent genes. Both the SA dependent and SA-independent functions of EDS1 and PAD4 contribute to plant basal immunity and effector-triggered immunity (ETI) (Rietz et al., 2011; Cui et al., 2017). These genes were also significantly induced in other recent transcriptional studies on HLB pathosystem (Fan et al., 2011; Mafra et al., 2013; Rawat et al., 2015).

Regarding downstream SA pathway genes, few significant alterations were observed, and most of the genes exhibited a down regulation expression (Table 2). NPR1 has been described as receptor of SA (Wu et al., 2012) and it was downregulated in this study (Table 2). NPR1 activity is regulated by proteasome mediated degradation, which is carried out by the NPR1 paralogues, NPR3 and NPR4. When SA levels are low, NPR4 maintain low NPR1 levels, but when SA levels increase, NPR4-NPR1 interaction is disrupted, leading the accumulation of NPR1. Furthermore, when SA level is extremely high, NPR3 binds NPR1 leading to NPR1 degradation (Spoel et al., 2009; Dempsey & Klessig, 2017). NPR1 turnover ensures a correct defense activation and the establishment of SA-induced responses (Spoel et al., 2009). NPR3 and NPR4 might be involved also in transcriptional regulation of SA-induced defense genes (Maruri-López et al., 2019). So, the level of NPR1 in the evaluated stages of CaL. infection is agreeing with high SA. Previous results has already showed down regulation of defense-related proteins in CLas-infected *C. lemon* (Nwugo et al., 2013), PR genes in CLas-infected sweet orange and rough lemon (*C. jambhiri*) (Fan et al., 2012), and NBS-LRR defense-related genes in sweet orange (Aritua et al., 2013).

Even with some minor differences in the gene expression profiles, it was not possible to establish a correlation between symptom development and systemic alteration in expression of SAR-related SA genes. Moreover, *P. trifoliata* plants exhibited even lower alteration in their gene expression profile. Therefore, regardless the noticeable fact that many important downstream pathway SA-genes are not active in diseased plants, it is not possible to assert the direct involvement of SA in citrus defense or susceptibility against HLB. SA-mediated defense seems not to be infallible because CaL. possibly have ways to overcome plant defense mechanism, as to direct lower SA accumulation by converting SA to inactive derivatives as MeSA, to interrupt SA biosynthesis by targeting specific pathways and to interfere with SA signaling (Qi et al. 2018). Li et al. (2017) showed that CLas may suppress plant defense by employing an active salicylate hydroxylase, thus halting SA accumulation and HR and allowing the pathogen to overcome the host defense.

Studies have shown that citrus plants display several physiological and cytological alterations in the phloem, including callose accumulation in response to CLas infection and hyperplasia in phloem parenchyma (Achor et al., 2010; Etxeberria et al., 2009; Pitino et al., 2016). In this study, we compared callose deposition and phloem alterations between healthy and CLas infected *C. sinensis* and *P. trifoliata*. Infected *C. sinensis* showed hypertrophy of the phloem parenchyma cells, promoting internal pressure in this tissue and leading to the collapse of the sieve tube elements (STE) (Figure 3a-b, 3d-e). Collapsed STE were not observed in *P. trifoliata* HLB infected (Figure 3j-k). Non-infected *C. sinensis* displayed few small and isolated fluorescent spots related to callose deposition (Figure 3c), whereas infected plants showed large callose deposits in several STE (Figure 3f). Koh et al. (2012) also observed similar levels of callose deposits in CLas symptomatic and asymptomatic leaves causing constriction, but not completely occlusion of sieve pores in infected STE. Base on the STE anatomy it was possible to recognize two different types of sieve plates: *C. sinensis* has simple sieve plates (Figure 3c) and *P. trifoliata* presents compound sieve plates (Figure 3i). This anatomical data could represent an important feature to minimize the effect of the phloem transportation caused by callose deposition in infected plants. Further ultrastructural analysis needs to be conducted to evaluate whether or not the compound sieve plates play any role in HLB tolerance or resistance.

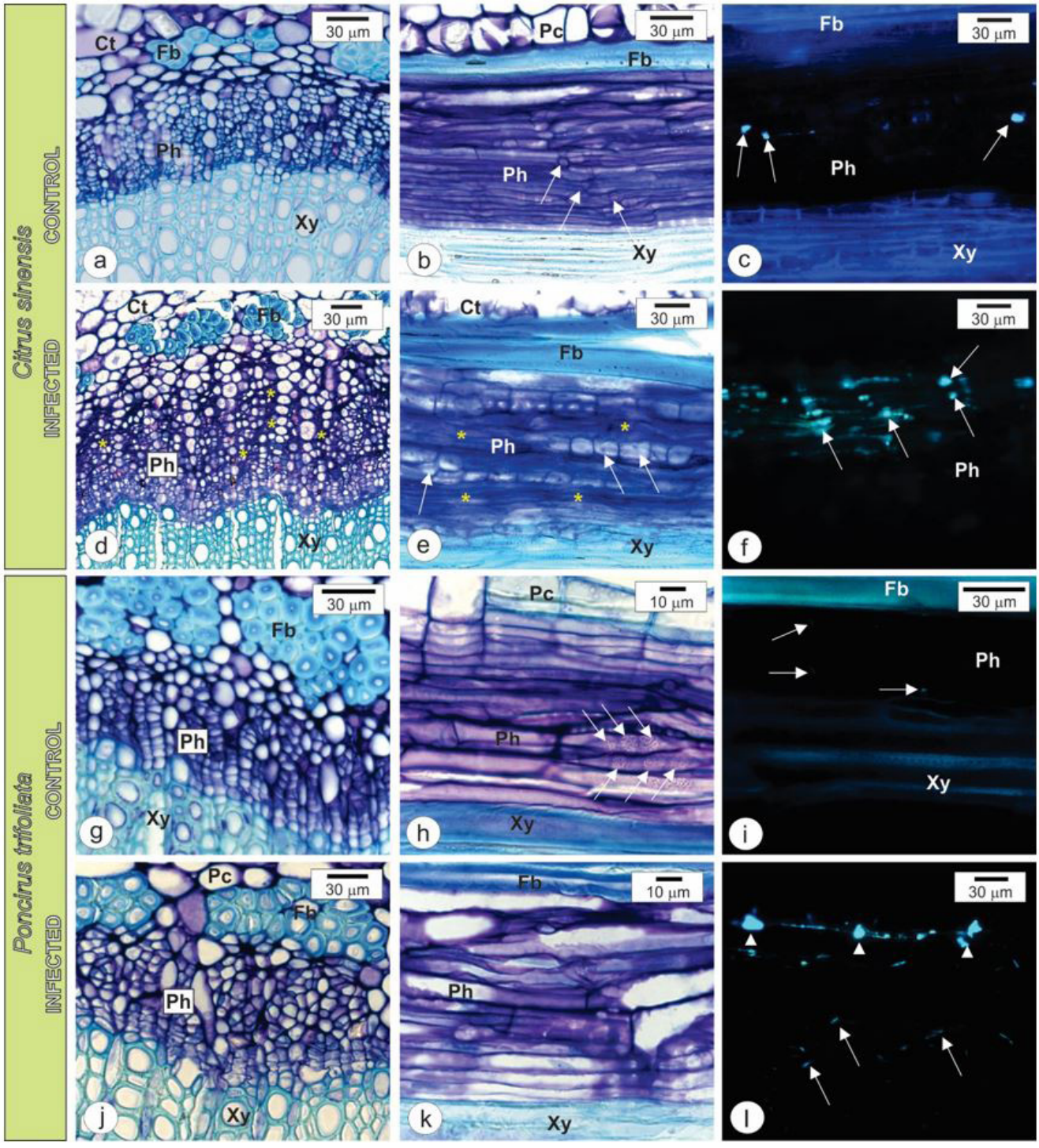

Figure 3. Phloem anatomy of control and CLas infected petioles of *Citrus sinensis* and *Poncirus trifoliata*. Cross sections (a, d, g, h); longitudinal sections (b-c, e-f, h-i, k-l); light microscopy (a-b, d-e, g-h and j-k); fluorescence microscopy after aniline blue staining method (c, f, l, l). In control was observed that *C. sinensis* present a simple sieve plate (arrows in b) and *P. trifoliata* exhibits compound sieve plates (arrows in h). *C. sinensis* infected plants show collapsed sieve tube elements (* in d-e). Hypertrophy of phloem parenchyma cells (arrows in e). Note the absence of collapsed cells in *P. trifoliata* (j-k). Callose deposition (arrows in c, f, I, l) occurs in all treatments but more evident in *C. sinensis* CLas infected. Note that *P. trifoliata* shows large amount of callose deposition associated to a single sieve tube (arrowhead in l). Ct – Cortex; Fb – Fiber; Ph – Phloem; Xy – Xylem.

In our study, callose deposition was observed in CLas infected *P. trifoliata* petiole (Figure 3g-h, 3j-k), albeit with lesser extent to that observed in *C. sinensis* (Figure 3l). There was also weak callose deposition observed in healthy *P. trifoliata* petiole, corroborating previous report (Boava et al., 2017). One important issue observed in *P. trifoliata* is that callose deposition is concentrated to few phloem tubes (Figure 3l). Nevertheless, if this difference contributes to the contrasting phenotype observed in the two rutaceous plants remain to be proven, but anatomical structure needs to be considered. Fan et al. (2012) compared the anatomical and transcriptional profile of one tolerant citrus species (rough lemon) with a susceptible one (sweet orange) infected by CLas and noticed callose accumulation and phloem collapse in both genotypes. Callose is a ubiquitous polysaccharide required for several developmental processes in higher plants (Chen & Kim, 2009). In addition, callose accumulation has been associated to immune-driven defense response against pathogen infection preventing host colonization (Boava et al., 2017; Luna et al., 2011).

Several studies have brought up some divergent aspects regarding callose-mediated defenses (Bonnemain et al., 2013; Ellinger et al., 2013). Arabidopsis mutants for an important callose biosynthesis associated gene *pmr4* (*POWDERY MILDEW RESISTANT4*) show hyperactivation of SA pathway, suggesting a negative regulatory action of callose in SA-mediated signal transduction (Eggert et al., 2014; Nishimura, 2008). In contrast, some researchers suggest that callose genes may be induced by SA (Dong et al., 2008).

Despite the recent progresses toward understanding the citrus - *Ca*. Liberibacter pathosystem, there is no conclusive model to explain the evolution of the HLB disease, especially regarding the pathogen-triggered events. Even though *Ca.* Liberibacter spp. are biotrophic pathogens, the systemic defense response mediated by salicylic acid seems to be inactive, or even compromised. Previous studies have indicated a SA-degrading enzyme coded by a gene present in CLas genome (Wang & Trivedi, 2013). Furthermore, Xu et al. (2015) reported a depression in SA pathway in CLas infected mandarin (*C. reticulata* Blanco cv. jiaogan). Our study corroborates those and suggests that possibly *Ca.* Liberibacter manipulate the plant defense response, converting SA to MeSA in order to suppress the defenses against it and to attract *D. citri*. Additionally, we detected the presence of callose deposition in the phloem of the susceptible *C. sinensis* upon CLas inoculation. However, less callose deposition was found in the tolerant/ resistant genotype *P. trifoliata*. Collapsed STE was observed only in sweet orange plants, suggesting that the occlusion of sieve elements could have a prominent implication to the impairment of phloem transport in susceptible citrus plants, what can at least partially explain the HLB symptoms. Another important factor observed here and that could be associated with *P. trifoliata* tolerance/ resistance to HLB is the anatomic differences of the sieve plates. Here, we suggest that the compound sieve plate could have a role in keeping the phloem function in infected plants.

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