Temporal Transcription Profiling of Sweet Orange in Response to PthA4-Mediated *Xanthomonas citri* subsp. *citri* Infection

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ABSTRACT

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Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, is a devastating disease of most commercial citrus varieties. In our previous study, we analyzed the transcriptional response of ‘Valencia’ sweet orange to *X. citri* subsp. *citri* wild-type and *pthA4* mutant infection at 48 h postinoculation (hpi). Using microarray analysis, two PthA4 targets, *CsLOB1* and *CsSWEET1*, were identified. We have shown that PthA4 binds to the effector binding element (EBE) of *CsLOB1* and activates gene expression of this susceptibility gene. However, how PthA4 modulates host genes at different stages of infection remains to be determined. In this study, we compared the transcriptional profiles between citrus leaf tissue inoculated with Xcc306 and those inoculated with a *pthA4*-deletion mutant strain (Xcc306Δ*pthA4*) at 6, 48, and 120 hpi. At both 48 and 120 hpi, the PthA4-mediated infection significantly upregulated expression of a variety of

genes involved in cell-wall degradation and modification, DNA packaging, G-protein, protein synthesis, sucrose metabolism, and cell division functions, while the downregulated genes were mainly enriched in photosynthesis, transport, secondary metabolism, cytochrome P450, and various plant defense-associated mechanisms. To validate microarray results, gene expression of 26 genes representing genes associated with cell-wall-associated, immunity system, and carbohydrate metabolism was confirmed using quantitative reverse-transcription polymerase chain reaction. Expression patterns of these genes at 48 and 120 hpi were consistent with the microarray results. We also identified putative EBE for PthA4 (EBEPthA4) in the promoter regions of multiple genes upregulated by PthA4, to which PthA4 might bind directly to control their gene expression. Our study provided a dynamic picture of citrus genes regulated by PthA4 during the *X. citri* subsp. *citri* infection of citrus leaves at different stages. This study will be useful in further understanding the virulence mechanism of *X. citri* subsp. *citri* and identifying potential targets of PthA4.

Additional keyword: transcriptome.

Citrus is one of the most economically important and extensively grown fruit tree crops worldwide (Boriss 2015). Citrus canker, which was originally identified in Southeast Asia and now is widely distributed in most citrus-producing countries, including Brazil, the United States, and China, poses a significant threat to the citrus industry. Citrus canker has caused huge economic losses due to reduced fruit quality and productivity, eradication programs, and regulatory restrictions.

Citrus canker affects all commercial citrus species and is caused by two phylogenetically distinct species of *Xanthomonas*: *Xanthomonas citri* subsp. *citri* and *X. fuscans* subsp. *aurantifolii* (Schaad et al. 2006). *X. citri* subsp. *citri* is the major pathogen in areas where citrus canker is present. *X. citri* subsp. *citri* is composed of strains that have been designated as A, Aw, and A* pathotypes, each with different host ranges. *X. fuscans* subsp. *aurantifolii* is grouped into pathotypes B and C. *X. citri* subsp. *citri* uses the type III secretion (T3S) system (Büttner and Bonas 2010; Laia et al. 2009) to deliver type III effectors to modify plant defense, acquire nutrients, cause typical canker symptoms, and assist bacterial release from the lesion (Brunings and Gabriel 2003). *X. citri* subsp. *citri* strain A (Xcc-A) and strain Aw (Xcc-Aw) contain 29 and 30 T3S effectors,

respectively (Escalon et al. 2013; Jalan et al. 2013), while *X. fuscans* subsp. *aurantifolii* strain B (Xfa-B) and strain C (Xfa-C) contain 27 and 26 T3S effectors, respectively (Moreira et al. 2010). Among all the type III effectors, PthA is a canker pathogenicity determinant and its homologs are present in all strains of the two *Xanthomonas* spp. that cause typical citrus canker disease (Cubero and Graham 2002; Swarup et al. 1992). Representative strains of the five different pathotypes that are responsible for citrus canker—Xcc-A, Xcc-A*, Xcc-Aw, Xfa-B, and Xfa-C—contain *pthA* homologs, which are designated *pthA*, *pthA**, *pthAw*, *pthB*, and *pthC*, respectively, and are essential for pustule formation on citrus (Al-Saadi et al. 2007). Transient expression of *pthA* in host plant cells was sufficient to induce citrus canker symptoms that include hypertrophy, hyperplasia, and necrosis (Duan et al. 1999). PthA and its homologs are transcription activator-like (TAL) effectors and contain nearly identical repeats of 34 amino acids in their central portion, an N-terminal T3S and translocation signal, a leucine zipper, three nuclear localization signal sequences, and an acidic transcriptional activation domain in the C terminus (Al-Saadi et al. 2007; Brunings and Gabriel 2003; Lahaye and Bonas 2001). PthA and its homologs are able to enter the plant nucleus and presumably activate host gene expression (Brunings and Gabriel 2003). Interestingly, de Souza et al. (2012) have identified PthA4-interacting proteins, including DNA or RNA-binding factors implicated in chromatin remodeling and repair, gene regulation, and mRNA stabilization or modification, further supporting the model of PthA4 being a TAL effector. TAL effectors bind to plant DNA elements, which are designated as effector binding elements (EBE), within the promoter regions via a series of amino acid repeats in the

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central coding portion (Boch et al. 2009; Hann et al. 2010; Moscou and Bogdanove 2009). The code for the interaction specificity between TAL effectors and EBE was cracked, indicating that the 12th and 13th repeat-variable diresidues determine the binding specificity of TAL and sequence of EBE (Boch et al. 2009; Moscou and Bogdanove 2009). Several TAL effector target prediction tools have been established for detecting the presence of EBE in gene promoters (Doyle et al. 2012; Grau et al. 2013; Pérez-Quintero et al. 2013).

Recently, we analyzed the transcriptional response of ‘Valencia’ sweet orange to *X. citri* subsp. *citri* wild-type (WT) and *pthA4* mutant (MU) infection at 120 hpi. The microarray data helped us identify two PthA4 targets, *CsLOB1* and *CsSWEET1*. We have shown that PthA4 binds to the EBE of *CsLOB1* and activates its gene expression. We demonstrated that *CsLOB1* is the major target of PthA4 and a citrus canker susceptibility gene (Hu et al. 2014). Zheng et al. (2014) also reported that *CsLOB* of citrus is targeted by PthA4. *CsLOB1* belongs to the lateral organ boundaries (LOB) domain family of transcription factor, which may modulate the downstream genes that contribute to the susceptibility. Multiple cell-wall-associated genes have been validated to be mediated by the expression of *CsLOB1* (Hu et al. 2014). However, in the previous study, we only analyzed the transcriptional response of Valencia sweet orange to *X. citri* subsp. *citri* WT and *pthA4* MU infection at 120 hpi. How PthA modulates host genes at different stages of

infection remains to be determined. In addition, the overall transcriptional effect of PthA4 on citrus was not analyzed in our previous study (Hu et al. 2014).

Microarray analyses of the transcriptional response of citrus to *Xanthomonas* spp. infection have been conducted using the citrus GeneChip. Cernadas et al. (2008) surveyed the early molecular events leading to canker development by performing transcriptional analysis of sweet orange plants infected with *X. citri* subsp. *citri* strain 306 (Xcc306) and Xfa-C strains, which elicit canker disease and the hypersensitive response, respectively. They observed that genes with significant expression changes in leaves after being infected by Xcc306 were involved in defense, cell wall modification and degradation, hormone response, vesicle trafficking, transcriptional regulation, and cell division. Fu and colleagues (Fu and Liu 2013; Fu et al. 2012) conducted two studies to investigate the global gene transcriptional changes after *X. citri* subsp. *citri* infection, by comparing the canker-resistant ‘Meiwa’ kumquat and the canker-resistant transgenic sweet orange with canker-susceptible WT sweet orange. Khalaf et al. (2011) revealed that varieties of genes associated with oxidative burst, protein degradation, and photosynthesis were differentially expressed in kumquat following the challenge of *X. citri* subsp. *citri*. In addition, the global transcriptional responses of citrus were analyzed following infection with the nonhost bacterium *X. campestris* pv. *vesicatoria* (Daurelio et al. 2013).

TABLE 1. Comparison of gene expression of sweet orange in response to wild-type (WT) and *pthA4* mutant (MU) of *Xanthomonas citri* subsp. *citri* via microarray (MA) and quantitative reverse-transcription polymerase chain reaction (PCR)

Probe ID ^b	Gene ID	Subject sequence description	Log FC (WT/MU) ^a			
			48 h		120 h	
			MA	PCR	MA	PCR
Cell wall						
<i>Cit.35756.1.S1_at</i>	...	Polygalacturonase, putative/pectinase, putative	4.727	5.247	5.044	9.421
<i>Cit.2949.1.S1_s_at</i>	<i>Cs2g14920.1</i>	Probable xyloglucan endotransglucosylase/hydrolase protein 5	5.819	4.629	4.104	7.4125
<i>Cit.13455.1.S1_s_at</i>	<i>Cs8g12020.2</i>	Probable xyloglucan endotransglucosylase/hydrolase protein B	4.514	5.455	4.53	6.3
<i>Cit.24058.1.S1_s_at</i>	...	Unknown protein	6.436	3.6795	5.089	7.2145
<i>Cit.7043.1.S1_at</i>	<i>Cs5g16960.1</i>	Trypsin and protease inhibitor family protein/Kunitz family protein	-5.916	-3.01	-4.651	-6.07
<i>Cit.30421.1.S1_s_at</i>	<i>Cs2g02100.2</i>	Cysteine protease inhibitor, putative/cystatin, putative	-4.114	-4.043	-0.922	3.016
<i>Cit.13956.1.S1_at</i>	<i>Cs3g27820.2</i>	Cyclin-dependent kinase B2-2	1.561	1.978	4.624	5.2175
<i>Cit.16483.1.S1_at</i>	<i>Cs1g26410.1</i>	G2/mitotic-specific cyclin-1	0.884	3.1255	4.069	5.262
<i>Cit.7588.1.S1_at</i>	<i>Cs2g29280.1</i>	G2/mitotic-specific cyclin-1	0.568	2.0215	4.633	3.732
<i>Cit.5864.1.S1_at</i>	<i>Cs6g16880.1</i>	G2/mitotic-specific cyclin-1	1.295	2.767	4.724	5.7515
<i>Cit.27164.1.S1_s_at</i>	<i>Cs3g20030.1</i>	Cyclin, N-terminal domain containing protein, expressed	2.112	0.396	4.716	5.2855
<i>Cit.7830.1.S1_at</i>	<i>Cs7g10190.1</i>	Mitotic spindle checkpoint protein MAD2	1.012	0.3815	4.501	5.191
<i>Cit.4137.1.S1_s_at</i>	<i>Cs2g30950.1</i>	Ubiquitin-conjugating enzyme E2 19	1.459	0.9675	5.151	5.246
Plant immunity						
<i>Cit.27302.1.S1_s_at</i>	<i>Cs4g06950.1</i>	FMO1 (flavin-dependent monooxygenase 1)	1.214	1.008	2.092	0.7835
<i>Cit.302.1.S1_s_at</i>	<i>Cs1g26330.1</i>	ATHCHIB (<i>Arabidopsis thaliana</i> basic chitinase)	1.038	0.866	1.253	0.843
<i>Cit.1200.1.S1_s_at</i>	<i>Cs5g10550.1</i>	Thaumatococcus-like protein	1.833	1.255	3.811	2.4825
<i>Cit.33130.1.S1_at</i>	<i>Cs2g05450.1</i>	MLO-like protein 6	-0.185	-0.33	0.419	0.0085
<i>Cit.6847.1.S1_at</i>	<i>Cs8g13710.1</i>	PR4 (pathogenesis-related 4)	-0.632	0.119	3.495	2.9505
<i>Cit.37394.1.S1_at</i>	<i>Cs3g12750.1</i>	DCD (development and cell death) domain-containing protein	0.085	2.5	0.006	-2.119
<i>Cit.25134.1.S1_at</i>	<i>Cs3g14240.1</i>	Respiratory burst oxidase homolog protein B	0.036	3.0425	0.604	-0.263
Carbohydrate						
<i>Cit.30272.1.S1_at</i>	<i>Cs8g01960.1</i>	sks5 (SKU5 similar 5)	4.862	-0.555	4.358	5.732
<i>Cit.14522.1.S1_at</i>	<i>Cs7g13250.2</i>	XYL1 (α -xylosidase 1)	4.499	0.641	4.031	5.897
<i>Cit.5370.1.S1_s_at</i>	<i>Cs9g14330.1</i>	Invertase/pectin methylesterase inhibitor family protein	7.719	1.354	8.088	8.7735
<i>Cit.4516.1.S1_s_at</i>	<i>Cs5g03170.1</i>	Probable polygalacturonase	5.036	-0.342	4.515	3.4745
<i>Cit.3554.1.S1_s_at</i>	<i>Cs5g20320.1</i>	CEL5 (cellulase 5)	7.016	0.3345	5.718	5.782
<i>Cit.13252.1.S1_at</i>	<i>Cs2g17090.2</i>	Endoglucanase 8	5.14	0.417	5.215	3.3795

^a FC = fold change of sweet orange in response to WT and *pthA4* MU of *X. citri* subsp. *citri*.

^b Genes related to cell wall, plant immunity, or the carbohydrate metabolic process.

Nevertheless, limited work has been done to examine temporal plant genome-wide transcriptional responses mediated by T3S effectors, which induce effector-triggered susceptibility or effector-triggered immunity. Truman et al. (2006) revealed that T3S effectors

TABLE 2. Citrus gene categories with significant expression change by the infection of wild-type Xcc306 (WT) relative to mutant Xcc306Δ*pthA4* (MU) at 6, 48, and 120 h postinoculation (hpi)

Functional categories	Probe number	P value
6 hpi WT versus MU		
Upregulated (NS) ^a	29	...
Downregulated	233	...
Auxin responsive	2	1.5 × 10 ⁻²
Acid and other phosphatases	3	9.5 × 10 ⁻³
48 hpi WT versus MU		
Upregulated	727	...
Cell wall	43	5.0 × 10 ⁻⁶
Cell wall degradation	11	3.9 × 10 ⁻⁵
Cell wall modification	15	1.7 × 10 ⁻⁶
Secondary metabolism-phenylpropanoids	3	8.0 × 10 ⁻³
Hormone metabolism-brassinosteroid	2	3.4 × 10 ⁻²
β-1,3 Glucan hydrolases	5	3.5 × 10 ⁻²
bZIP transcription factor family	4	4.3 × 10 ⁻²
DNA synthesis/chromatin structure	31	1.2 × 10 ⁻⁴
Protein synthesis	15	4.0 × 10 ⁻³
Ribosomal protein synthesis	14	9.1 × 10 ⁻³
Signaling in sugar and nutrient	2	4.0 × 10 ⁻²
Downregulated	584	...
Photosynthesis	24	4.4 × 10 ⁻⁴
Light reaction	19	1.0 × 10 ⁻³
Stress	37	1.1 × 10 ⁻⁸
Stress-biotic	30	4.9 × 10 ⁻¹⁰
Stress-biotic, pathogenesis-related proteins	25	2.6 × 10 ⁻¹¹
Posttranslational modification in kinase	3	3.8 × 10 ⁻²
Signaling receptor kinases	8	1.1 × 10 ⁻²
Receptor kinases-DUF 26	3	8.6 × 10 ⁻³
Development	14	3.0 × 10 ⁻²
120 hpi WT versus MU		
Upregulated	913	...
Cell wall	53	2.9 × 10 ⁻³
Cell wall degradation	16	1.4 × 10 ⁻²
Cell wall modification	16	3.1 × 10 ⁻²
Glycolipid synthesis	3	3.8 × 10 ⁻²
FA synthesis	2	2.5 × 10 ⁻²
Auxin responsive	5	1.9 × 10 ⁻²
Redox regulation	6	6.4 × 10 ⁻³
Development	23	3.7 × 10 ⁻³
Transport	26	5.5 × 10 ⁻⁴
Sugar transporter	3	2.0 × 10 ⁻²
Metabolite transporter	2	3.6 × 10 ⁻²
Downregulated	1,094	...
Photosynthesis	73	5.8 × 10 ⁻²
Light reaction	56	1.7 × 10 ⁻²
Cell wall	31	2.9 × 10 ⁻³
Cell wall AGPs proteins	4	1.5 × 10 ⁻²
Cell wall pectin methyl esterase	3	1.5 × 10 ⁻²
Lipid metabolism	31	9.0 × 10 ⁻³
FA synthesis	11	2.5 × 10 ⁻³
Lipid transfer proteins	4	1.7 × 10 ⁻²
Stress	21	4.4 × 10 ⁻²
Redox, glutaredoxins	5	2.8 × 10 ⁻²
Miscellaneous enzymes	117	4.4 × 10 ⁻⁴
Lipid transfer protein	7	2.8 × 10 ⁻²
GDSL-motif lipase	17	3.0 × 10 ⁻⁵
Nitrile lyases	10	1.2 × 10 ⁻²
RNA	50	2.0 × 10 ⁻⁴
Regulation of transcription	46	2.3 × 10 ⁻³
TCP transcription factor	5	4.6 × 10 ⁻²
AP2/ERF transcription factor	4	4.7 × 10 ⁻²
Aux/IAA family	4	1.3 × 10 ⁻²
Protein	43	1.9 × 10 ⁻²
Posttranslational modification	9	3.8 × 10 ⁻²
Minor CHO metabolism	14	4.4 × 10 ⁻²
Light signaling	7	8.9 × 10 ⁻³
Receptor kinase	18	4.9 × 10 ⁻²

^a NS = no significant category.

suppressed the expression of genes in categories of leucine-rich repeat receptor-like kinase, primary carbon metabolism, aromatic biosynthesis, and phenylpropanoids and induced MYB, NAC, and AP2 transcription factor genes in *Arabidopsis*, by comparing the invasion of *Pseudomonas syringae* pv. *tomato* DC3000 with that of T3S effector delivery deficient MU strain DC3000 *hrp*-. AvrBs3 of *X. campestris* pv. *vesicatoria* was reported to induce auxin-induced genes, α-expansin gene, pectate lyase, and anthocyanidin rhamnosyl transferase genes in pepper (Marois et al. 2002). Balaji et al. (2007) observed that 139 genes and 1,294 genes had significant changes in transcript levels at 8 h and 12 h postinoculation (hpi), respectively, following inoculation of isogenic *X. campestris* pv. *vesicatoria* strains differing only in the *avrXv3* gene.

In this study, we further analyzed how PthA4 modulates host genes at different stages of bacterial infection by investigating the transcriptional response of Valencia sweet orange to *X. citri* subsp. *citri* WT and *pthA4* MU infection at 6, 48, and 120 hpi. We provided the overall transcriptional effect of PthA4 on citrus in this study. The microarray result has been validated using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). In addition, we searched the putative EBE_{PthA4} and target genes in the pool of genes upregulated by PthA4.

MATERIALS AND METHODS

Plant materials and bacterial strains. Valencia sweet orange (*Citrus sinensis*) plants were kept in a quarantine greenhouse facility at the Citrus Research and Education Center, Lake Alfred, FL. The temperature ranged between 25 and 30°C with a 12-h photoperiod. Before inoculation, the plants were pruned to stimulate new leaves. The leaves chosen for infiltration were fully expanded, immature leaves, 14 to 21 days old. The plasmids and bacterial strains used in this study are list in Supplementary Table S1. Strains of *Xanthomonas* were grown at 28°C on nutrient agar (NA), *Escherichia coli* was grown at 37°C in lysogeny broth.

Microarray experiment and analyses. Xcc306 WT strain and Xcc306Δ*pthA4* MU were cultured on NA plates and used to inoculate Valencia sweet orange at the concentration of 5 × 10⁸ CFU/ml suspended in sterile tap water. The suspensions were infiltrated with a needle and syringe into the entire leaf, then kept in a growth chamber for the duration of the experiment. Leaves were harvested 6, 48, and 120 hpi for RNA isolation. We compared the global transcriptome profiles of citrus with the infections of the two near-isogenic strains using microarray analysis. To allow stringent statistical data analysis, three biological replicates were conducted and three leaves were pooled for each replicate per time point. RNA extraction was performed by using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Microarray analysis was conducted using an Affymetrix array containing 30,171 probesets representing up to 33,879 citrus transcripts. Labeling, hybridization, washing, scanning, and data analysis were performed at the ICBR (Interdisciplinary Center for Biotechnology Research) facility at University of Florida in Gainesville. Statistical tests were performed using BioConductor statistical software, an open source software based on the R programming language (<http://www.bioconductor.org/>). A robust multichip analysis approach was used for normalizing the raw data. Differential expression analysis was carried out using a linear modeling approach and the empirical Bayes statistics implemented in the Limma package. Differentially expressed genes with an adjusted *P* value (false discovery rate [FDR]) ≤ 0.05 were considered to be differentially expressed genes at a statistically significant level. Differentially expressed genes were defined as genes with an expression fold change ≥ 3.0 (|log fold change| of 1.58) in comparison with leaves inoculated with WT and MU strains, and that were statistically significant at an adjusted *P* value ≤ 0.05. The microarray data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) and are accessible through GEO accession number GSE52700 and

GSE50741. We compared the differentially expressed genes at 6, 48, and 120 hpi to observe the dynamics of PthA4-mediated responses, and analyzed the citrus genes that changed in mRNA level in response to WT infection between the three time points. We further assigned the differentially expressed genes into functional categories according to their similarity to genes with known functions in plants using two distinct programs, MapMan software (Thimm et al. 2004; Usadel et al. 2005) and the web-based singular enrichment analysis (SEA) tool in the AgriGO program (Du et al. 2010).

qRT-PCR analyses. The RNA was subjected to DNase I treatment and first-strand cDNA synthesis by using the ProtoScript AMV First-Strand cDNA Synthesis Kit (NEB, Ipswich, MA). Two-step

real-time PCR was performed using RealMasterMix SYBR Rox (5 PRIME, Gaithersburg, MD). The gene-specific primer sequences are listed in Supplementary Table S2. The elongation factor (EF) gene *EF1α* was used as endogenous control. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification (Livak and Schmittgen 2001).

Categorization of differentially expressed genes. For MapMan analysis, the microarray data of each comparison with log twofold change value and FDR value were loaded into the software with a filter of FDR < 0.05. The pathways were visualized in the context of built-in *Citrus* AFFY mapping, and a Wilcoxon rank sum test implemented in MapMan was used to extract the item number in each BIN and return BIN whose gene members exhibited a

Fig. 1. Comparison of PthA4 upregulated and downregulated genes of citrus by wild-type versus mutant *Xanthomonas citri* subsp. *citri* strain infection at 48 h postinoculation based on MapMan analysis. Bar chart showing the categories with upregulated genes (white bars) and downregulated (black bars).

Fig. 2. Visualization of PthA4 upregulated genes and downregulated citrus genes in different categories by wild-type versus mutant *Xanthomonas citri* subsp. *citri* strain infection at 48 h postinoculation using MapMan software. The scale with $\log_2$ fold change is shown.

significant difference in expression. To analyze gene ontology (GO) term enrichment of differentially expressed genes, SEA was performed online through the agriGO program, a GO analysis tool kit for the agricultural community (<http://bioinfo.cau.edu.cn/agriGO>). Briefly, the differentially expressed gene probe identification numbers were first uploaded into agriGO; then, the Citrus Affymetrix Genome Array was selected as the reference, statistical *P* values were calculated using the Fisher method, and multiple-test adjustment was performed using the Yekutieli (FDR under dependency) method. The Venn diagrams were generated using an online program, Draw Venn Diagram (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

Fig. 3. Venn diagram showing the number of upregulated (black font) or downregulated (red font) genes modulated at 6, 48, and 120 h postinoculation following wild-type strain Xcc306 inoculation relative to mutant strain Xcc306Δ*pthA4* inoculation. The genes with fold change no less than 3 and adjusted *P* value ≤ 0.05 were considered up- or downregulated.

RESULTS

Validation of microarray data using qRT-PCR. To validate the microarray results, 26 citrus genes were tested for their expression at 48 and 120 hpi using qRT-PCR. The qRT-PCR results suggested that the expression patterns of most genes were consistent with the microarray results (Table 1).

Characterization of differentially expressed genes following inoculation of WT relative to MU strain at 6, 48, and 120 hpi. We employed the MapMan Wilcoxon Rank Sum Test to classify the differentially expressed genes and calculate the significance based on whether the probability that the differentially expressed genes assigned to a BIN or sub-BIN (function category) were statistically different from all other BIN or sub-BIN (*P* value < 0.05). At 6 hpi, inoculation of WT in comparison with inoculation with MU strains resulted in 29 genes being induced and 233 citrus genes being suppressed. No category showed a significant difference for the upregulated (WT or MU) genes, whereas only two categories showed significant differences for the downregulated genes (Table 2). We found that, at this time point, the WT strain containing *pthA4* downregulated a high percentage of genes sorted into the categories of transport, biotic stress, signaling (primarily receptor kinase), miscellaneous enzymes, RNA regulator, and secondary metabolism compared with MU strain Xcc306Δ*pthA4* (Supplementary Fig. S1).

At 48 hpi, 1,311 differentially expressed genes were identified, which accounted for 4.35% of the total transcripts in the citrus GeneChip. Among them, 727 genes were upregulated and 584 genes were downregulated by WT infection compared with MU strain infection. According to the MapMan Wilcoxon Rank Sum Test, the main significantly upregulated categories were related to cell wall degradation and modification, DNA synthesis or chromatin structure, and ribosomal protein synthesis; while the processes related to plant defense response, photosynthesis, development,

Fig. 4. Over-represented categories in upregulated genes by infection of Xcc306 relative to mutant Xcc306Δ*pthA4* at 48 h postinoculation in singular enrichment analysis. Input (dark gray bar) represents the percentage out of total upregulated genes and reference (light gray bar) represents the percentage of genes in an individual category out of total citrus expressed-sequence tag number. Categories with input significantly exceeding reference are thought to be over-represented (false discovery rate < 0.05). Genes were categorized based on biological process (upper), molecular function (bottom left), and cellular component (bottom right).

stress-related pathogenesis-related (PR) proteins, and receptor kinase signaling were the significantly downregulated categories (Table 2). Furthermore, we also examined the proportions of each annotated category. The categories of cell wall, cell (cell cycle and cell division), DNA, signaling, and transcription factor contained mostly upregulated genes (Supplementary Fig. S2), while the categories of miscellaneous enzyme families, secondary metabolism, biotic stress, photosynthesis, and transport contained the highest percentage of downregulated genes. When comparing the up- and downregulated genes in different categories, the categories of photosynthesis, secondary metabolism, hormone metabolism, biotic stress, cytochrome P450, transport, glucosyl, and glucuronyl transferase, which were enriched for downregulated genes, contained only limited numbers of upregulated items, while categories associated with cell wall, glucosidase and galactosidase, DNA, protein synthesis, nucleotide metabolism, G-protein signaling, and cell (cell cycle and cell division) contained a large number of upregulated genes and only a few downregulated genes (Fig. 1). We also displayed the differentially expressed genes and the corresponding categories in a MapMan visualized pathway diagram (Fig. 2), which showed the same affiliations as in Figure 1. However, the functions of a large number of differentially expressed genes were unassigned (42% for upregulated and 37% for downregulated genes), which shared no reliable homology to genes deposited in public databases and were without known functions.

At 120 hpi, 913 and 1,094 citrus genes were up- and downregulated, respectively, after comparing WT infection with MU strain infection. A large number of functional categories exhibited significant expression changes, the pattern was similar with that at

48 hpi (Table 2; Supplementary Fig. S3) in MapMan analysis. The Mercator analysis showed that cell wall, cell (cell cycle and cell division), and DNA categories contained the highest proportion of upregulated genes (Supplementary Fig. S4A), while the proportions of photosynthesis and secondary metabolism categories were high in the downregulated genes (Supplementary Fig. S4B).

The overlapping differentially expressed genes at the three time points were compared by Venn diagram analysis. No gene was found to be upregulated by PthA4 at all three time points (Fig. 3). Only one gene, *Cit.57.1.S1_x_at*, which encodes a protease inhibitor protein, was identified to be downregulated at all three time points. A dramatic difference was observed for the transcriptional profiles at 48 and 120 hpi, despite the overlapping genes.

GO annotation of differentially expressed genes in citrus following inoculation by WT compared with MU strain at 6, 48, and 120 hpi. In AgriGO-SEA interpretation, the enrichment or over-representation of a category was assessed with a significantly (FDR < 0.05) higher percentage in total differentially expressed genes (input in Figures 4 and 5) than its overall percentage in the total expressed sequence tag (reference in Figures 4 and 5). At 6 hpi, the downregulated genes were enriched in the categories of innate immune response, oxidoreductase activity, carbohydrate binding, carboxylic acid biosynthesis, and ubiquitin ligase (Supplementary Table S3).

At 48 hpi, the over-represented categories of upregulated genes included DNA (protein-DNA complex assembly, DNA packaging, and DNA replication), cell wall (cell wall organization and modification), metabolism (carbohydrate, sucrose, and starch

Fig. 5. Over-represented categories in downregulated genes by infection of Xcc306 relative to mutant Xcc306Δ*pthA4* at 48 h postinoculation in singular enrichment analysis. Input (dark gray bar) represents the percentage out of total downregulated genes and reference (light gray bar) represents the percentage of genes in an individual category out of total citrus expressed-sequence tag number. Categories with input significantly exceeding reference are thought to be over-represented (false discovery rate < 0.05). Genes were categorized based on biological process (upper), molecular function (bottom left), and cellular component (bottom right).

metabolic process), microtubule-based movement, protein polymerization, and trichome differentiation based on biological processes. The upregulated genes were also enriched in pectate lyase activity, pectinesterase activity, DNA-dependent ATPase activity, motor activity, copper ion binding, and enzyme regulator activity based on molecular function; and enriched in cell wall, endomembrane system, cytoplasmic vesicle, cytoskeleton, extracellular region, and protein-DNA complex based on cellular component (Fig. 4). For the downregulated genes, with regard to biological process, they were primarily enriched in phenylpropanoids, L-phenylalanine, monocarboxylic acid, amino acid, and lipid metabolism processes; and electron, peptide, and carbohydrate transport. In molecular function, the over-represented categories of downregulated genes were catalytic activity, transport, enzyme inhibitor, and ion and heme binding, while they were also enriched in cell wall, photosystem, thylakoid, and organelle subcompartment based on cellular component (Fig. 5).

TABLE 3. Categories with significant change in expression (P value < 0.05) and differentially expressed probe set numbers through the comparisons of different times after infiltration of wild-type Xcc306 generated by MapMan software

Function categories ^a	Probe number	P value
48 versus 6 hpi		
Upregulated	1,147	...
Cell wall	38	7.5×10^{-10}
Cell wall degradation	13	1.5×10^{-4}
Cell wall modification	16	3.1×10^{-8}
Stress	75	1.8×10^{-2}
Biotic stress	32	1.4×10^{-2}
Heat stress	33	4.3×10^{-2}
Nitrile lyases	8	5.2×10^{-3}
MYB-related transcription factor	6	1.2×10^{-2}
C2C2 (Zn)-like zinc finger family	5	3.4×10^{-2}
DNA synthesis	28	2.9×10^{-5}
Ribosomal protein synthesis	15	4.1×10^{-2}
Signaling	59	3.6×10^{-3}
G-protein signaling	11	1.2×10^{-2}
Transport	51	1.7×10^{-3}
Downregulated	1,119	...
Cell wall modification	4	1.5×10^{-2}
Lipid transfer proteins	4	4.5×10^{-2}
Lysophospholipases	6	1.2×10^{-2}
Secondary metabolism-isoprenoids	19	5.0×10^{-2}
Secondary metabolism-chalcones	6	1.4×10^{-2}
Thiamine metabolism	7	2.1×10^{-2}
Stress	53	1.2×10^{-3}
Biotic stress	32	7.5×10^{-3}
Pathogenesis-related protein	23	1.4×10^{-3}
Proteinase inhibitors	17	5.8×10^{-4}
Peroxidases	10	3.7×10^{-3}
WRKY domain transcription factor family	5	4.6×10^{-3}
RNA binding	6	1.3×10^{-3}
Light signaling	3	5.3×10^{-3}
120 versus 48 hpi		
Upregulated	468	...
Cell	27	1.9×10^{-2}
Cell cycle	9	1.2×10^{-2}
Development	17	1.3×10^{-2}
Late embryogenesis abundant	3	1.3×10^{-2}
Downregulated	552	...
Cell wall	24	1.6×10^{-2}
Cell wall AGPs protein	4	3.8×10^{-3}
Cell wall modification	5	1.4×10^{-3}
Cell wall pectin methyl esterase	3	1.7×10^{-3}
Lipid metabolism	18	5.3×10^{-4}
FA synthesis	9	2.7×10^{-3}
Biotic stress	10	5.7×10^{-3}
Oxidases	5	3.9×10^{-2}
Cell	5	1.4×10^{-2}
Calcium transport	3	4.5×10^{-2}

^a Abbreviation: hpi = hours postinoculation.

Most of the differentially expressed genes at 48 and 120 hpi had similar distribution patterns in AgriGO-SEA categorization. Moreover, oxidoreductase activity and lyase activity categories over-represented the downregulated genes at all three time points.

Reprogramming of citrus gene expressions at different time points after *X. citri* subsp. *citri* inoculations. We compared the genome-wide expressions in citrus at 6, 48, or 120 hpi following the infection by WT Xcc306, given the diversity of the differentially expressed genes in different time points. Xcc306 induced 1,147 and repressed 1,119 citrus genes at 48 hpi when compared with 6 hpi. Based on MapMan Wilcoxon Rank Sum analysis, the significantly upregulated categories included cell wall degradation and modification, heat stress, nitrile lyase, MYB transcription factors, DNA synthesis, ribosomal protein synthesis, signaling, G-protein, and transport; and the significantly downregulated categories included cell wall modification, lipid transfer proteins, isoprenoid metabolism, thiamine metabolism, PR protein, peroxidases, WRKY transcription factor family, RNA binding, and light signaling (Table 3). We also contrastingly and visually checked the differentially expressed genes in a MapMan Pathway diagram, which revealed that categories of cytochrome P450, terpenes, flavonoids, phenylpropanoids, peroxidase, ethylene, and jasmonic acid contained remarkably more downregulated genes than upregulated genes, while classes of calcium, G-protein, cell wall, cell cycle, cell division, and auxin were enriched with more upregulated genes. Furthermore, we also performed an AgriGO-SEA test to interpret this time-based comparison (Supplementary Table S4). It illustrated that most of the differentially expressed gene-enriched categories were consistent with that in the comparison of WT versus MU strain at 48 hpi (Tables 2 and 3).

In addition, we conducted a comparative analysis between 48 and 120 hpi following the WT infection. The number of differentially expressed genes in respect to this comparison was less than that of the 48 versus 6 hpi comparison (Table 3), and the significantly upregulated categories were mainly related to development. Based on the AgriGO-SEA study, the upregulated genes were enriched in categories of chitin catabolic process, starch and sucrose metabolic, cellular nitrogen compound metabolic, microtubule-base movement, mitosis, response to oxidative stress, cell cycle, cell division, peroxidase activity, chitinase activity, peptidase inhibitor activity, and heme binding, while the main, significantly downregulated categories included glucan metabolic process, lipid and peptide transport, response to auxin stimulus, developmental growth, monooxygenase activity, and carboxylesterase activity.

Candidate target genes that contain a potential EBE_{PthA4} box in promoter region. In terms of the gene activation ability and DNA-binding specificity of PthA4, the potential EBE box from promoters of the PthA4-induced genes at three time points were searched. For this purpose, the upstream 1,000 bp before the 5' untranslated region of these induced genes was extracted from the published sweet orange genome annotation (Xu et al. 2013) using a custom script and three distinct programs: Target Finder (Moscou and Bogdanove 2009), TALgetter (Grau et al. 2013), and TALVEZ (Pérez-Quintero et al. 2013) were employed to identify the EBE box. The optimized sequence of EBE_{PthA4} is supposed to be TATAAACCTCTTTACCTT, and only the potential EBE starting with a T that precedes the first repeat were taken into consideration. As expected, at 48 and 120 hpi, the gene represented by *Cit.37210.1.S1_at*, which encodes CsLOB1, ranked number 1 in all three programs. At 48 hpi, 15 genes contain putative EBE_{PthA4}, whereas 9 genes contain putative EBE_{PthA4} at 120 hpi. Four common genes were predicted to contain EBE_{PthA4} at 48 and 120 hpi (i.e., *Cit.37210.1.S1_at*, *Cit.5726.1.S1_at*, *Cit.30096.1.S1_at*, and *Cit.15320.1.S1_at*). No potential EBE_{PthA4} was detected for the upregulated genes at 6 hpi (Supplementary Table S5). Target Finder and TALgetter retrieved a similar gene pattern, whereas some of the target genes predicted by TALVEZ were different (Table 4).

DISCUSSION

In this study, we used microarray analysis to scrutinize the reprogramming of the transcription profiles in sweet orange following PthA4-dependent bacterial infection. Our study provided a dynamic picture of citrus genes regulated by PthA4 during the *X. citri* subsp. *citri* infection of citrus leaves at different stages. This study will be useful in further understanding the virulence mechanism of *X. citri* subsp. *citri* and identifying potential targets of PthA4.

In Xcc306, PthA4 is the only TAL effector that is required and sufficient to reproduce typical citrus canker (Al-Saadi et al. 2007; Brunings and Gabriel 2003); therefore, how it exerts its functions inside plant tissues is critical for disease development and bacterial growth in planta. As a typical TAL effector, PthA4 presumably plays important roles in activating gene transcription, suppressing the plant defense system, and promoting plant vulnerabilities, which are supported by our microarray analysis of plant response to *X. citri* subsp. *citri* infection with or without PthA4. We revealed that, at 6 hpi, PthA4 downregulated expression of genes involved in the plant innate immunity. Suppression of plant defense is important for bacterial pathogens to colonize the intercellular spaces (Dangl et al. 2013). At the later stages (48 and 120 hpi), the presence of PthA4 was necessary to induce expression of genes involved in various processes and functions (e.g., susceptibility and release of bacterial cells) and, meanwhile, inhibited expression of genes associated with plant defense response and plant development. Marois et al. (2002) studied host genes induced by AvrBs3, a TAL effector present in *X. campestris* pv. *vesicatoria*, by performing a cDNA-amplified fragment length polymorphism analysis and identified 13 genes which mainly include auxin-induced and expansin-like genes. The host genes induced by AvrBs3 are suggested to be

involved in hypertrophy development. Similarly, we also identified auxin-induced and expansin-like genes induced by PthA4, which suggests that both AvrBs3 and PthA are necessary for hypertrophy development. Two TAL effectors of *X. oryzae* pv. *oryzae* (i.e., PthXo1 and AvrXa7) activate sugar transporter genes *SWEET11* and *SWEET14* to promote sugar transport in the host plants to supply some of the nutritional needs of the bacterial pathogen (Antony et al. 2010; Chen et al. 2010; Yang et al. 2006). PthA4 upregulated genes in the signaling in sugar and nutrient category at 48 hpi and in the sugar transporter category at 120 hpi, which suggests that PthA4 might have similar functions in promoting sugar transport for the nutritional needs of the pathogen (Table 2).

Furthermore, at 48 and 120 hpi, PthA4 was shown to be required for repressing genes in several categories that were known to play positive roles in plant defense. Plant cytochrome P450, which is a group of heme-containing monooxygenases, has been reported to be associated with the synthesis of plant defense compounds and defense against microbial pathogens (Fu et al. 2012; Hwang and Hwang 2010; Khalaf et al. 2007). UDP-glycosyl transferases, which catalyze the transfer of nucleotide-diphosphate-activated sugars to secondary metabolites, play positive roles in plant defense against pathogens (Langlois-Meurinne et al. 2005; Vogt and Jones 2000). Plant defense components which are defined as the secondary metabolism, including PR proteins, phenylpropanoids, terpenes, and flavonoids, prevent pathogen invasion and are generally considered to be indicators for defense responses to biotic stimuli (Fu and Dong 2013; Sels et al. 2008). T3S effectors have been reported to repress plant defense by targeting key modules of plant immune signaling pathways such as immune receptor complexes and mitogen-activated protein kinase cascades either acting as

TABLE 4. Putative targets of PthA4 deduced from the upregulated genes at 48 h postinoculation predicted with three different programs

Sequence ID	Program, ranking ^a	Distance to 5' UTR ^b	Target sequence	LFC ^c	Putative functions description
Optimized	N/A	N/A	T ATAAACCTCTTTTACCTT	N/A	
<i>Cit.37210.1.S1_at</i>	Target Finder, 1	42	T ATAAACCCCTTTTGCCCTT	6.592	CsLOB1, lateral organ boundaries domain family
	TALgetter, 1	...	...	...	...
	TALVEZ, 1	...	...	...	...
<i>Cit.31052.1.S1_at</i>	Target Finder, 2	761	T CTAAACCTCTTTGACCGT	2.17	Mitochondrial glycoprotein family protein
	TALgetter, 2	...	...	...	...
	TALVEZ, 3	...	...	...	...
<i>Cit.5726.1.S1_at</i>	Target Finder, 3	442	T TAAAACCTCTGTAACTT	1.856	Hypothetical protein
	TALgetter, 3	...	...	...	...
<i>Cit.17510.1.S1_at</i>	Target Finder, 4	626	T ATAAACCCCTTATTCTTT	1.758	Succinyl-CoA ligase, α 1 subunit
	TALgetter, 7	...	...	...	...
	TALVEZ, 4	...	...	...	...
<i>Cit.15813.1.S1_at</i>	Target Finder, 5	277	T CAGAAACTCTTTTCCCTT	2.021	Unknown protein
	TALgetter, 4	...	...	...	...
	TALVEZ, 2	...	...	...	...
<i>Cit.30096.1.S1_at</i>	Target Finder, 6	835	T ACTAACTTATTTAACCTT	3.902	Remorin family protein
	TALgetter, 6	...	...	...	...
<i>Cit.37603.1.S1_at</i>	Target Finder, 7	449	T TCAAACAGATTTTACCTT	1.903	Transcription factor S-II domain-containing protein
	TALgetter, 5	...	...	...	...
<i>Cit.6643.1.S1_at</i>	Target Finder, 8	422	T AAAAAGGTCTTTTCCCTA	2.225	Ras-related protein Rab7
<i>Cit.12835.1.S1_s_at</i>	Target Finder, 9	452	T CCTCACCCCTTTCCCTT	1.889	Ubiquitin extension protein
	TALgetter, 9	...	...	...	...
	TALVEZ, 8	...	...	...	...
<i>Cit.6643.1.S1_at</i>	Target Finder, 10; TALgetter, 8	400	T AGTAACTGCTTTTCCCTT	2.225	Ras-related protein Rab7
<i>Cit.11546.1.S1_at</i>	TALgetter, 10	98	T GCAAATCTCTTTTACCT	1.886	Octicosapeptide/Phox/Bem1p domain-containing protein
	TALVEZ, 5	...	...	...	...
<i>Cit.15320.1.S1_at</i>	TALVEZ, 6	661	T AGCCCCATCTTCTTCCTC	3.93	Cysteine-rich polycomb-like protein
<i>Cit.37086.1.S1_s_at</i>	TALVEZ, 7	471	T ACCAAACACTCTCTCCTC	2.847	Remorin family and DNA binding protein
<i>Cit.26969.1.S1_s_at</i>	TALVEZ, 9	168	T GCCCACCTCTCATTCATT	2.218	β -Galactosidase
<i>Cit.8050.1.S1_s_at</i>	TALVEZ, 10	854	T GTCCAACCCCTTTAACCTT	2.175	Tubulin/FtsZ family protein

^a N/A = not applicable.

^b UTR = untranslated region.

^c LFC = log₂ fold change.

enzymes (e.g., acetyltransferase, ADP-ribosyltransferase, cysteine protease, E3 ligase, protein tyrosine phosphatase, phosphothreonine lyase, and SUMO protease) or by physical association and interference (Axtell and Staskawicz 2003; Espinosa and Alfano 2004; Fu et al. 2007; Hotson et al. 2003; Janjusevic et al. 2006; Kim et al. 2009; Li et al. 2007; Shao et al. 2003; Tasset et al. 2010). How PthA4 exerts its effect on plant defense remains to be investigated.

As a typical TAL effector, PthA4 presumably targets host genes by binding to the EBE directly (Hu et al. 2014; Li et al. 2014). The known TAL effector target genes in planta, without exception, were highly induced by the corresponding TAL effectors (Antony et al. 2010; Gu et al. 2005; Kay et al. 2007; Yang et al. 2006; Yu et al. 2011). Interestingly, AvrBs3, another TAL effector of PthA/AvrBs3 family, has been shown to bind to a conserved element in the promoter of *upa20*, a master regulator of cell size, via its central repeat region and induces gene expression through its activation domain (Kay et al. 2007). Recently, we have identified two targets of PthA4 (i.e., *CsLOB1* and *CsSWEET1*) that contain EBE_{PthA4} and are highly induced by PthA4. We demonstrated that *CsLOB1* is the major target of PthA4 and a citrus canker susceptibility gene (Hu et al. 2014). Interestingly, we have identified multiple genes upregulated by PthA4 in addition to *CsLOB1* containing sequences similar to EBE_{PthA4} in their promoter regions, although with low identity (Table 4). Whether PthA4 could directly interact with the promoters of those candidate genes and whether they are involved in modulating expression of plant defense-related genes need further elucidation.

Interestingly, dramatic transcriptional differences were observed for citrus to PthA4 at 6, 48, and 120 hpi of *X. citri* subsp. *citri* infection. One potential explanation for the differential expression of citrus to PthA4 at different time points is that *X. citri* subsp. *citri* may use PthA4 to regulate different sets of host genes at different infection stages (e.g., in early and late infection stages). Alternatively, this could be a cascade of events involving indirect regulation by PthA4 based on earlier events where PthA4 is critical. The fewer genes regulated at 6 hpi compared with 48 and 120 hpi indicate the time needed for *X. citri* subsp. *citri* to translocate PthA4 into the plant nucleus and, consequently, result in induction of citrus genes. PthA4 activated various genes involved in cell division, which is associated with hypertrophy and hyperplasia at 48 and 120 but not at 6 hpi. Hypertrophy and hyperplasia have been suggested to play important roles in bacterial growth and ultimate release from citrus canker lesions for further transmission (Brunings and Gabriel 2003; Yan and Wang 2012). Additionally, PthA4 upregulated many genes involved in cell wall modification at 48 or 120 hpi (Table 2). This expression pattern is consistent with pustule symptoms of citrus canker. The cell wall modification-associated genes (e.g., cell wall disassembly enzyme encoding genes) are involved in the increase in plant vulnerability (Cantu et al. 2008), which further benefits *X. citri* subsp. *citri* in planta. Intriguingly, the categories of DNA replication, synthesis, and packaging, which are involved in protein-DNA assembly, were significantly induced at 48 and 120 hpi. This is probably due to the fact that PthA4 requires the assistance of a protein-DNA interaction complex to activate the target genes.

Collectively, by dissecting the compendium of citrus host transcripts and their dynamics affected by PthA4-dependent Xcc306 infection, we can gain insight into the chronology of molecular events in citrus susceptibility to Xcc306 and identify the most critical elements in citrus for disease initiation and development that are largely unknown. Our study indicated that *X. citri* subsp. *citri* employs PthA4 to regulate a suite of host genes to benefit *X. citri* subsp. *citri*. Furthermore, this study allows us to exploit and identify major genes that are potentially responsible for citrus canker disease development, which could be used in molecular breeding to improve plant resistance to citrus canker. This data could provide useful information to explore the mechanisms of plant disease development mediated by other TAL effectors.

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