CITRUS CANKER REQUIRES TARGETING OF A SUSCEPTIBILITY GENE BY SPECIFIC TAL EFFECTORS PRESENT IN XANTHOMONAS CITRI

By

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To my parents for their unconditional love and support

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LIST OF ABBREVIATIONS

AD	Activation domain
BA	Brassinosteroids
bHLH	Basic helix-loop-helix
cfu	Colony forming units
DE GENES	Differentially expressed gene
dTALes	designed TAL effectors
EBEs	Effector Binding Elements
ET	Ethylene
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
GA	Gibberellin acid
GUS	Glucuronidases
GO	Gene ontology
hpi (dpi)	Hours (days) post inoculation
hrp	HR and pathogenicity
HR	Hypersensitive response
JA	Jasmonates
MU	*pthA4* deletion mutant of Xcc306, Xcc306Δ*pthA4*
NLS	nuclear localization signal
PR	Pathogenesis related
PTI	PAMP-triggered immunity
qRT-PCR	Quantitative Real-Time PCR
R GENE	Resistance gene
RVD	Repeat-variable diresidue

S GENE	Susceptible gene
SA	Salicylic acid
SEA	Singular Enrichment Analysis
TAL	Transcription activator like
TALEs	TAL effectors
TALEN	TAL effector nuclease
TF	Transcription factor
vs.	Versus
WT	Wild-type Xcc306
Xcc	*Xanthomonas. citri* ssp. *citri*
Xfa	*Xanthomonas. fuscans* ssp. *aurantifolii*

Abstract of Dissertation Presented to the Graduate School
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Citrus canker is a devastating disease that infects many citrus varieties and causes huge economic loss. The causal agents are members of a complex of distinct yet related strains of *Xanthomonas*. *X. citri* subsp. *citri* strain Xcc306 harbors four members of the type III transcription activator like (TAL) effector gene family, one of which (*pthA4*) is required for pustule formation. Transcriptional profiles were compared between citrus leaves tissue infected with Xcc306 and with the *pthA4*-defective strain Xcc306Δ*pthA4*, it revealed that a considerable number of genes involved in cell wall modification, DNA packaging, and cell division functions were up-regulated by PthA4-mediated infections, while the down-regulated genes were enriched in photosynthesis and various plant defense associated categories. Two genes, *CsLOB1* and *CsN3-1*, which were induced in a PthA4-dependent manner, contain predicted effector binding elements (EBE) of PthA4 in their respective promoters. *CsLOB1* is a member of the lateral organ boundaries (LOB) domain gene family of transcription factor, and *CsN3-1* is a homolog of the sugar transporter and disease susceptibility gene family of rice. PthA4 was shown to drive expression of *CsLOB1* and *CsN3*-1 promoter reporter gene fusions when co-expressed in citrus or in *N. benthamiana*. Artificially designed TAL

effectors (dTALes) specifically directed to sequences in the *CsLOB1* promoter region, but not the *CsN3-1* promoter, promoted pustule formation and higher bacterial leaf populations. Four additional distinct TAL effector genes, *pthAw*, *pthA*, pthB* and *pthC*, also restored pustule formation and directed the expression of *CsLOB1*, while *pthB* and *pthC* unlike *pthAw, pthA** did not promote expression of *CsN3-1*. PthAw and PthA* share the same EBE as PthA4, while EBEs of PthB and PthC in *CsLOB1* promoter were found to be located 6 bp upstream of EBE_{PthA4}. The results indicated that diverse citrus canker-inciting species of *Xanthomonas* exploit TAL effectors target a single disease susceptibility gene in the host by altering the expression of this otherwise developmentally regulated gene. The discovery of TAL effectors common target in citrus provides new approaches to control citrus canker.

CHAPTER 1
GENERAL INTRODUCTION

Citrus Canker Disease

Overview of Citrus Canker

Citrus is one of the most valuable fruit crops shown to be the good source of vitamin C. U.S. is the third largest citrus producer in the world. In 2010, the United States exported and imported $2.9 billion and $3.7 billion worth of citrus fruit and products (Boriss and Huntrods, 2011). The citrus industry is a major constituent of Florida's agricultural economy, which accounted for 63 percent of the total U.S. citrus production in 2010, and was valued at about $ 9 billion in 2007-2008 season (Boriss and Huntrods, 2011; Rahmani and Hodges, 2009). Citrus canker is a major threat to citrus growers especially in Florida. Citrus canker is a disease originally identified in Southeast Asia and now is distributed worldwide. It is a major disease on citrus in Asia, Africa and the Americas. Citrus canker has caused huge economic damages, which is a significant threat to the citrus industry. The economic impact of citrus canker comes from the loss of citrus quality and productivity, the eradication program cost and most importantly, the regulatory restrictions on the shipment of fruit. In Florida, citrus canker outbreaks and eradications program have occurred sporadically. For example, the last extensive outbreak involving type A strains occurred from 1995 through 2006, triggering an, ultimately, unsuccessful eradication program that cost an estimated US $1 billion; in Brazil, up to 1998 over half a million canker contaminated trees have been destroyed. Citrus canker affects all citrus species, while some species are more susceptible than others, with grapefruit (*C. paradise*) and key lime being (*C. aurantiifolia*) the most susceptible cultivars, while sweet orange (*C. sinensis*) is relatively tolerant (Gottwald et

al., 2002). In another aspect, their vulnerabilities also depend on the host ranges of the invasive pathogens.

Causal Agents of Citrus Canker

There are two phylogenetically distinct species of *Xanthomonas* causing citrus canker, *X. citri* ssp. *citri* (Xcc) and *X. fuscans* ssp*. aurantifolii* (Xfa). Each group contains several subgroups basd on host range (Brunings and Gabriel, 2003; Schaad et al., 2006). The most widespread species is Xcc which is further distinguished into A, A^w and A* strains according to their host ranges. Type A strain of Xcc causes disease on most species of citrus, whereas type A^w and type A* strains are restricted to Key lime (Verniere et al., 1998; Sun et al., 2004). Studies showed that T3SS effector AvrGf1 was only present in all A^w and some A* strains, which demonstrated that it may contribute to the exclusion of grapefruit from the A^w or A* host strains (Rybak et al., 2009; Escalon et al., 2013). The second species Xfa is grouped into type B and type C, the diseases they cause are named as Canker B and Canker C. Canker B was originally identified in Argentina which is only present in Argentina, Paraguay, and Uruguay and is mostly restricted to lemon (*C. limon*) (Civerolo, 1984), whereas Canker C is limited to the state of São Paulo, Brazil with host range being restricted to key lime (Malavolta et al., 1984). The reasons for their host range limitations are still unknown, whereas an avirulence gene with homology to AvrGf1 may be account for the incompatible response (unpublished data). What's more, some other undescribed variants of *Xanthomonas* may also exist that cause citrus canker. The host ranges of all these groups and their relative pathogenicities on different citrus cultivars are shown in Table 1-1.

Symptoms and Control of Citrus Canker

All of the above *Xanthomonas* strains generate very similar citrus canker symptoms. The pathogens infect citrus tissue through stomata and wounds, while they are disseminated by wind, rain, leaf miner and nursery plant. On leaves, the symptom is leaf spot; the young lesions are raised or have a pustule appearance on both surfaces. These pustules will become corky with raised margins and a sunken center. A characteristic symptom of the disease on leaves is the yellow halo that surrounds lesions. On stems and fruit, the symptoms are raised corky lesions surrounded by water soaked margins. In heavily infected trees it causes defoliation and premature fruit drop. Nowadays, the management and control of citrus canker are mainly through integrated approaches including windbreaks, eradication programs and copper-based chemical control (Gramham et al., 2004).

General Principles of Bacterial Pathogen and Plant Host Interaction

Type III Secretion System

Bacterial plant pathogens use various strategies to infect the host, and then interfere with the host cellular pathways to favor its growth and multiplication. Among these strategies, one of the most important ones is the type III secretion system (T3SS). Most of the Gram-negative plant pathogenic bacteria including xanthomonads cause disease by injecting a repertoire of effectors into plant cells via the T3SS.

The T3SS is encoded by the *hrp* (HR and pathogenicity) gene cluster which consists of more than 20 genes. It include the extracellular Hrp pilus as the key secretion apparatus which spans both bacterial and host membranes and serves as transport channels for secreting effector proteins into the host. The *hrp* genes that were conserved with their siblings in animal pathogens are also called *hrc* genes

(hypersensitive response and conserved). *hrp* gene expression is regulated by diffusible signal molecules (DSFs), cyclic di-GMP levels, regulator HrpG and HrpX, while the inactivation of *hrp* genes leads to the loss of pathogenicity and reduction of bacterial survival (Büttner and Bonas, 2010). There are two types of T3SS, including the flagellar T3SS, which secretes extracellular flagellum components, and the translocation-associated T3SS which secretes effector proteins. Here, we focus on the latter one. The translocation of effectors into host-pathogen interface is dependent on the assembly of the channel-like translocon and the regulatory systems, which form a hierarchical process (Deane et al., 2010; Buttner, 2012).

Characteristics of Type III Effectors Especially That from *Xanthomonas*

Type III effectors are delivered to suppress plant basal defenses, damage plant cellular processes to favor the pathogen growth and promote disease development. The number of effectors is variable between different bacteria. Within *P. syringae*, 62 effectors were identified while each strain encodes 20-30 effectors (http://www.pseudomonas-syringae.org/) (Studholme et al., 2009). Among them, the most intensively studied effector is AvrPtoB, which contains a kinase binding domain and an E3 ubiquitin ligase domain; it suppresses PTI by targeting the receptor kinase FLS2 and co-factor BAK1 (for flagellin) or by targeting CERK1 (for chitin) (Abramovitch et al., 2006; Gohre et al., 2008; Shan et al., 2008).

In *Xanthomonas* species, there are about 64 known effectors (http://www.xanthomonas.org), but each individual *Xanthomonas* strain only secretes a battery of about 15 or more effectors, most of which achieve a lot of functional redundancy and individually are dispensable but collectively indispensable and interchangeable (Hann and Rathjen, 2010). In the case of Xcc-A and Xcc-A^w, both

contain twenty-four and thirty putative T3S effectors, respectively, while Xfa-B and Xfa-C, contain twenty-seven and twenty-six T3S effectors, respectively (Moreira et al., 2010). Most sequenced *Xanthomonas* spp. encode a core set of nine type III effectors (XopR, AvrBs2, XopK, XopL, XopN, XopP, XopQ, XopX and XopZ) except *X. campestris* pv. *armoraciae* which has only six known effectors and *X. albilineans* which contains no identified effectors (Ryan et al., 2011). Several types of T3SS effectors in xanthomonads have been shown to play important roles in pathogenicity, localizing to multiple compartments and manipulating the host for their benefit. The collection of these T3SS effectors are designated as *Xanthomonas* outer proteins (Xop). It has been determined that some of them have enzymatic functions that played important roles in the interaction with the host (Kay and Bonas, 2009). AvrBs2 in *Xanthomonas campestris* pv*. vesicatoria* was the first T3SS effector shown to enhance bacterial multiplication in the host tissue and contribute to virulence (Kearney and Staskawicz, 1990). After that, a lot of effectors in *Xanthomonas* were identified to support the virulence of the bacteria (Kay and Bonas, 2009). In *X. campestris* pv*. vesicatoria*, XopX promoted lesion development and restricted basal plant defenses in *Nicotiana benthamiana* (Metz et al., 2005); XopD was required for full virulence and could suppress the expression of defense-related genes by targeting and hydrolyzing SUMO proteins through its cysteine protease activity (Kim et al., 2008); the Type III effector XopJ from *X. campestris* pv*. vesicatoria*, belongs to the SUMO peptidases, and was shown to inhibit cell-wall associated defense response by affecting vesicle trafficking and protein secretion (Bartetzko et al., 2009); XopN was revealed to interact with a tomato atypical receptor-like kinase (TARK1) to interfere with the defense process

including callose deposition (Kim et al., 2009a); the expression of *XopZ*$_{PXO99}$ in *Xanthomonas oryzae* pv. *oryzae* could interfere with host innate immunity (Song and Yang, 2010).

Plant Molecular Responses to the Invasion of Bacterial Pathogens

Generally, plants respond to pathogen infections by deploying a complicated innate immune system. Basal disease resistance has been defined as the resistance activated by virulent pathogens on susceptible host, which include PAMP-triggered immunity (PTI) and weak effector triggered immunity (ETI) triggered by weak recognition but exclude effector-triggered susceptibility (ETS) (Chisholm et al., 2006; Jones and Dangl, 2006). The first phase occurs in the infection process and is referred to as PTI. In PTI, transmembrane pattern recognition receptors (PRRs) in the plant sense microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) such as bacterial flagellin or elongation factor (EF-Tu) and halts the infection. In the second phase, the pathogens can interfere with PTI by deploying virulence effectors, which results in ETS. Then the effector might be specifically recognized by direct or indirect interaction with polymorphic NB-LRR proteins encoded by *R*-genes directly or indirectly, resulting in ETI and disease resistance, and usually results in a hypersensitive response (HR) at infection sites. Evolutionarily, the pathogens acquire additional effectors or shed the recognized ones by natural selection to dodge the surveillance system and suppress ETI, but also the plants can acquire new *R* genes and trigger ETI again.

Goals and Objectives of This Study

In this study, all four *pthA* homology genes in Xcc306 were examined for their function in causing citrus canker. Until now, only *pthA4* that is most similar to *pthA* was confirmed to be required for pathogenicity; however, the functions of the other effectors

are still poorly understood. In order to dissect the genes and biological processes in citrus that are specifically affected by the delivery of bacterial TAL effector PthA4, microarray analyses were carried out to monitor the differential expression of genes by comparing the infection of Xcc306 and *pthA4* deficiency strain; these genes were functionally categorized. Given that no evidence is available relating to the time points in PthA4 activating gene expression in planta, several time points after inoculation were sampled for the microarray assay. This, may further provide solid evidence for identifying the genes and processes involved in disease development. So the compendium of citrus host transcripts and their dynamics that affected by Xcc306 infection especially by PthA4-mediated challenge can be illustrated. Most importantly, the genes specifically activated and targeted by TAL effector PthA4 will be identified based on the predicted and unique binding sequences in the gene promoter region. Collectively, by conducting this study, we aim to get insight into mechanisms of citrus susceptibility to Xcc306 and the most critical elements in citrus for disease initiation and development that yet are largely unknown. In one aspect, the discovery of the PthA4 targets and downstream elements will contribute as an addenda to the known mechanisms of TAL effector-based susceptibility and a reference for further TAL effector research; in another aspect, it supplies great gene resources to engineer and generate transgenic plants controlling the citrus canker disease, from both the suppressed defense genes and the induced susceptible genes.

Table 1-1. Reaction of all known citrus canker-causing xanthomonad strains on four different Citrus species

xanthomonad groups	Relative pathogenicity in Sweet orange	Grapefruit	Key lime	Lemon
Xcc-A	++	++++	++++	+++
Xcc-A^{w}	-/HR	HR	++++	-
Xcc-A*	-	-	++++	-
Xfa-B	+	+	++++	+++
Xfa-C	HR	HR	++++	HR

+, weak citrus canker disease; ++++, strong citrus canker disease; -, no symptoms; HR, hypersensitive reaction

CHAPTER 2
MUTAGENESIS ANALYSIS OF PTHA HOMOLOGY GENES IN XCC306

Background Information

Xanthomonas spp. use most of the general strategies as other bacterial pathogens to invade the citrus host. As a result of sequencing the Xcc strain 306 genome (da Silva et al., 2002), mutagenesis and bioinformatics analysis revealed many genes in *Xanthomonas* that are involved in the citrus canker disease development; these include genes coding for type III secretion systems (T3SS), T3SS effectors, the quorum-sensing system, extracellular polysaccharides (EPS), lipopolysaccharide (LPS), and type II secretion systems (T2SS) (Brunings and Gabriel, 2003; Moreira et al., 2010; Yan and Wang, 2012). Some of the factors control the growth and multiplication of the bacterium in planta, some determine the colonization and invasion into plant cells, and others interrupt the triggered plant defense systems.

Type III secretion systems. Type III secretion systems (T3SS) were widely proven to be essential for the delivery of virulence factors. In T3SS, it showed that HrpG and HrpX regulons, which regulate all hypersensitive response and pathogenicity (*hrp*) genes, played significant roles in pathogenesis, since the mutations in the *hrpG* or *hrpX* genes in Xcc resulted in loss of pathogenicity on citrus (Laia et al., 2009; Figueiredo et al., 2011a; Guo et al., 2011). Some other T3SS genes, like *hrpA*, *hpaB*, *hrcV* and *hrcN* were also identified as important factors in symptom formation in these studies.

T3SS effectors. A lot of T3SS effectors act as pathogenicity determinant which are required for promotion or suppression of canker disease development. The most important and widely studied ones are PthA and its homologs (Brunings and Gabriel, 2003), which belong to the AvrBs3/PthA family and were characterized as TAL effectors

(transcription activator like) that were able to activate host gene expression. Some of them have been shown to play critical roles in virulence. Maker-exchange mutagenesis of *pthA* gene in Xcc resulted in a complete loss of virulence on citrus (Swarup et al., 1991). Exogenous expression of *pthA* gene in other xanthomonads conferred the ability to elicit canker symptoms on citrus (Swarup et al., 1992), while transient expression of *pthA* inside the host cells was also sufficient for inducing citrus canker symptoms including hypertrophy, hyperplasia and cell death (Duan et al., 1999). The *pthA* homolog, *hssB3.0*, from Xcc strain KC21 was responsible for suppression of virulence on *Citrus grandis* (Shiotani et al., 2007). In Xcc306, four *pthA* genes were described and determined to be located on two distinct plasmids pXAC33 and pXAC64 (da Silva et al., 2002); however, using EZ-Tn5 insertion mutagenesis-based high-throughput screening, only *pthA4* was shown to be important for the citrus canker happening, while the other homologs, *pthA1*, *pthA2*, *pthA3* were not identified by the screen to be involved in virulence of the pathogen (Yan and Wang, 2012). Two additional T3SS effectors in Xcc306 and Xfa, XopA1 and XopE3, may also play a special role in citrus canker (Hajri et al., 2009; Moreira et al., 2010). Two effectors named AvrXacE1 and AvrXacE2 from Xac99-1330 decreased plant tissue necrosis by interrupting different signaling pathways (Dunger et al., 2012). But the mechanism of how these effectors contribute to host-bacterium interaction is still elusive.

EPS and LPS. EPS and LPS have been shown to be the important virulence factors for many bacterial pathogens. The *opsX* gene, which affects the biosynthesis of EPS and LPS, was reported to play a role in virulence on plant (Kingsley et al., 1993). The *gum* gene cluster is responsible for the xanthan biosynthesis and hence for

virulence of many xanthomonads (Katzen et al., 1998). A mutant of *gumB* showed defective production of xanthan and reduced disease symptoms in lemons (Rigano et al., 2007). *gumD* mutants displayed impaired epiphytic survival of the bacteria although they remained pathogenic (Dunger et al., 2007). A knockout of the *galU* gene brought a reduction in EPS level and no symptoms in grapefruit (Guo et al., 2010). Furthermore, the *wxacO* and *rfbC* genes of Xcc encode proteins with a role in LPS biosynthesis and the following virulence (Li and Wang, 2011). In the high-throughput screening of Xcc306, two *gum* genes (*gumF* and *gumK*) were shown to be involved in virulence in grapefruit, since both the *gum* mutants reduced water-soaking and bacterial cell densities. What's more, four genes (*wzt*, *wzm*, *rfbC* and *XAC3599*) that are associated with LPS biosynthesis were identified to play critical roles in plant-pathogen interactions (Yan and Wang, 2012).

Type II secretion systems. The bacterial T2SS has been shown to mediate the transportation of diverse molecules such as toxins and plant cell-wall degrading enzymes including protease, amylase, pectate lyase, cellulase, and xylanase (Russel, 1998), which contribute to the aggressiveness of the bacterium. In Yan et al.'s (Yan and Wang, 2012) screening, six T2SS-related genes (*xpsD*, *xpsM*, *xpsN*, *xpsE*, *xpsF*, and *xpsG*) were identified to potentially contribute to the virulence of Xcc306, with mutations of these genes causing delayed symptom development in host plants.

Quorum sensing system. Many bacteria use quorum sensing (QS) system to accomplish cell-cell communication and coordinate certain behaviors based on the cell density. In *X. campestris* pv. *campestris*, the QS system is established by the production of diffusible signal factor (DSF), and DSF biosynthesis is modulated by two

Rpf proteins, RpfF and RpfC, which form a two-component system. QS system plays an important role in citrus canker pathogenicity, possibly by regulating the production of EPS and extracellular enzymes, including proteases and cellulases (He and Zhang, 2008). The mutation of quorum-sensing sensor kinase gene *rpfC* abolished pathogenicity in grapefruit (Yan and Wang, 2012).

Plant natriuretic peptide-like protein. Only Xcc but no other pathogenic bacterium has been shown to contain a plant natriuretic peptide-like gene (*xacPNP*) (Gottig et al., 2008). PNP proteins are a class of extracellular, systemically mobile peptides that elicit a number of important plant responses in homeostasis and growth. The *xacPNP* deletion mutant resulted in more necrotic tissues, earlier bacterial cell death and more pronounced decrease in photosynthetic proteins than infected with the wild-type strain, which suggests that XacPNP enables Xcc to modify host response in order to accommodate its lifestyle and create favorable reservoirs for its survival and further colonization (Garavaglia et al., 2010). Because of the uniqueness, *xacPNP* might be one of the genes contributing to a high level of virulence displayed by Xcc.

Others Virulence factors. Some other elements used by Xcc for the infection of citrus include: 1) adhesion proteins for the initial stage of infection process such as XacFhaB and XacFhaC (Gottig et al., 2009); 2) regulatory factors, e.g. *dksA* which encodes an expression regulator of ribosomal RNA; and 3) transporters, i.e. ATP-binding cassette (ABC) transporter system. As more complete genome sequences available for citrus canker-causing bacteria, i.e., Xfa-B and Xfa-C strains (Moreira et al., 2010), Xcc-A^w strain 12879 (Jalan et al., 2013), through genome comparisons, the

mechanisms for virulence and host range of citrus canker-causing bacteria will be further characterized basing on their different pathogenicities.

Here, we focus on the *pthA* homologs in Xcc306, to investigate their function in causing citrus canker.

Results

Construct of *pthAs* Deletion Mutants from Xcc306

To investigate the AvrBs3/PthA family genes from Xcc306 involved in citrus canker, the four copies of *pthA* genes were knocked out one by one by marker exchange with double crossover; the created mutants were designated as Xcc306Δ*pthA1*, Xcc306Δ*pthA2*, Xcc306Δ*pthA3*, and Xcc306Δ*pthA4* respectively. In addition, considering the high probability of reduced virulence for *pthA4* deletion mutant, three double mutants Xcc306Δ*pthA1*Δ*pthA2*, Xcc306Δ*pthA1*Δ*pthA3*, Xcc306Δ*pthA2*Δ*pthA3* and a triple mutant, Xcc306Δ*pthA1*Δ*pthA2*Δ*pthA3* were also constructed to determine if *pthA4* alone was sufficient for pustule formation. Then the quadruple mutants Xcc306Δ*4pthA* with all four of the *pthAs* deleted was created. The mutants were confirmed using both PCR and Southern blot, the mutants were shown to contain either shorter or missing bands (Figure 2-1A and B).

Pathogenicity Test of the Mutant Strains

The mutant strains were tested for the ability to produce pustule as well as the ability to grow in planta. In both sweet orange and grapefruit, only *pthA4* deletion mutant Xcc306Δ*pthA4* showed extraordinarily weaker pustule formation than that by wild-type Xcc306, while the mutant could be complemented back by the expression of *pthA4* through the plasmid bearing *pthA4* (Figure 2-2A and B); these results were in agreement with previous observation in a high-throughput virulence-deficient strains

screening (Yan and Wang, 2012). Interestingly, the triple mutant Xcc306Δ*pthA1*Δ*pthA2*Δ*pthA3* developed here showed no reduction in pustule forming ability (Figure 2-2), indicating that only *pthA4* was required and sufficient for pustule formation on citrus. To assess the ability of Xcc306Δ*pthA4* growing in planta, the bacterial growth dynamics were tested with two inoculum densities, 5×10^8 colony forming units (cfu) /ml and 5×10^5 cfu/ml, in both sweet orange and grapefruit. Intriguingly, no significant population differences were detected between Xcc306 and Xcc306Δ*pthA4* in both sweet orange and grapefruit when inoculated at 5×10^8 cfu/ml (Figure 2-3A and B). However, when the initial inoculum concentration was 5×10^5 cfu/ml, lower populations were observed for Xcc306Δ*pthA4* than for Xcc306 after 4 dpi (days post inoculation) in sweet orange, but in grapefruit, small differences were observed in cell densities between these two strains (Figure 2-3C and D), which was in sharp contrast with previous reports that *pthA* mutants showed extremely lower cell density than that of the wild-type (Swarup et al., 1991; Yan and Wang, 2012).

Materials and Methods

Plant Material, Bacterial Strains, and Plasmids

Plants of grapefruit (*C. paradise*), cultivar Duncan, sweet orange (*C. sinensis*), cultivar Valencia, and Key lime (*C. aurantifolia*) were kept in a glasshouse in Gainesville, Florida. The temperature ranged between 25 and 30 °C and a 12/12 h photoperiod. Before inoculation, the plants were pruned to stimulate new leaves. The leaves chosen for infiltration were 14-21 days old fully expanded new leaves. The plants were kept in the growth room at 28 ± 2 °C with 16 h light and 40-60% humidity after the inoculation of bacteria. The plasmids and bacterial strains used in this study are list in Table 2-1. Strains of *Xanthomonas* were grown at 28 °C in nutrient agar (NA),

Escherichia coli was grown at 37 °C in lysogeny broth (LB). The antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; rifamycin SV, 100 µg/ml; spectinomycin, 100 µg/ml, tetracycline 12.5 µg/ml, gentamicin 10 µg/ml and chloramphenicol, 30 µg/ml.

Mutagenesis of *pthAs* in Xcc306

To delete *pthA* genes from the genome DNA of Xcc306, *pthA* genes including about 600 bp flank sequences were amplified; the four PCR reactions were run under the same touchdown protocol: 1 cycle of 4 minutes at 95°C; 15 cycles of 35 seconds at 95°C, 40 seconds per cycle starting at 68°C, decreasing setpoint temperature after cycle 2 by 1°C per cycle, and extension for 5 minutes at 72°C; 18 cycles of 35 seconds at 95°C, 40 seconds at 54°C and extension for 5 minutes at 72°C; and an additional extension for 10 minutes at 72°C. The central regions of the PCR products were deleted by *Bam*H I and self-ligated (Figure 2-4). The deleted *pthA* genes fragments were excised with *Apa* I and *Spe* I from pGEMT clone for ligation into suicide vector pOK1. Restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase (Promega, Madison, WI, USA) were used according to the manufacturer's recommendations. Through triparental (donor, recipient and helper) conjugation, the *pthA*-knocked out strains were produced as described by Huguet et al. ((Huguet et al., 1998), with the mutations being labeled Xcc306Δ*pthA*. For the double, triple and quadruple mutants, single, double, and triple mutants were used as recipients respectively. The mutants were confirmed by PCR with single *pthA* specific primers and Southern blot with universal *pthA* probe. The Southern blot was accomplished by Dr. Becky Bart in the lab of Dr. Brian J. Staskawicz at the University of California-Berkeley. The primers used were list in Table 2-2. For the

complement, *pthA4* gene containing the up-stream and down-stream sequence was amplified and ligated into the *Xanthomonas* expression vector pLARF3, the construction pLARF3:pthA4 was delivered into Xcc306Δ*pthA* through triparental conjugation.

Bacterial Inoculation and Growth Assay in *planta*

The bacteria were diluted to defined concentrations and infiltrated into leaf tissues using syringe with needle, and kept in growth chamber. For the population of bacteria strains in citrus plants, one leaf disc with 1 cm^2 of the inoculated area was taken and macerated in sterile tap water, after serial dilutions, 50 µl were plated on NA medium and incubated at 28 °C for 3 days, the colony counts were calculated to determine the internal populations. This experiment was repeated three times.

Discussion

In this study, a critical pathogenicity determinant factor, PthA4, was identified via mutagenesis analyses, and the *pthA4* defective mutant had reduced pathogenicity in citrus species sweet orange and grapefruit. PthA4 was also found to be the only TAL effector in Xcc306 to be responsible for aggressiveness and disease development. Interestingly, the mutant Xcc306Δ*pthA4*, which had reduced pathogenicity, was able to multiply in *planta* at almost the same level as wild-type when inoculated at high cell densities (5×10^8 cfu/ml), whereas population of the mutant was significantly lower 4 days after infiltration at the lower concentration of 5×10^5 cfu/ml in sweet orange, while in grapefruit the differences were not obvious. This phenomenon was dissimilar with what was previously reports conducted in grapefruit. In the study conducted by Swarup et al. (Swarup et al., 1991), the *pthA* insertion mutant had a sharp decline in bacterial population as compared to wild type on grapefruit, but the complemented strain did not restore the bacterial growth; the cell density of another *pthA4* insertion mutant created

by Yan et al. (Yan and Wang, 2012) also showed 20 fold-lower than that of wild-type in grapefruit when inoculated at low concentration. One plausible explanation is that in the bacterial growths in planta relies on the status of host plant and surrounding environment factors when in the absence of PthA4.

It is not surprising when regarding the non-function in pathogenicity of the three *pthA* homology genes; it is very common for TAL effectors to have undescribed functions in other *Xanthomonas* strains. Nineteen TAL effectors have been identified in the *X*. *oryzae* pv. *oryzae* strain PXO99^{A}; only four of them are associated with virulence or avirulence by activating *S* genes to induce disease or *R* genes to cause HR phenotype (Salzberg et al., 2008). A *pthA4* homologue, *avrTaw*, did not restore symptom of the pustule-minus strain that originated from Xcc-A^{w}, while *pthAw* did (Rybak et al., 2009). One reasonable explanation for the appearances of these redundant TAL effectors is that they act as reservoir to evolutionally generate new TAL effectors for subverting plant defense and adaptation to new hosts through recombination or horizontal gene transfer. Another hypothesis is that they may contribute to the host range since the Xcc group A strains have wider host range than most of the other groups that contain less *pthA* homologs, but all of four *pthAs* from Xcc strain 3213 that resemble four *pthAs* in Xcc306 did not increase the host range of A* strain when they were conjugated into this A* strain (Al-Saadi et al., 2007). Recently, some other citrus proteins in addition to α-importin that mediate the transfer to nucleus were found to interact with PthAs. By yeast two-hybrid screening of citrus cDNA library, PthA2 and PthA3 were reported to preferentially target a citrus cyclophilin (Cyp), a tetratricopeptide domain-containing thioredoxin (TDX), ubiquitin-conjugating enzyme

Uev and Ubc13, which were associated with protein folding, k63-linked ubiquitination, transcription control and DNA damage repair (Domingues et al., 2010). The same group also discovered that PthA4 interacts with proteins in chromatin remodeling and repair, gene regulation and mRNA stabilization/modification that involved in translational control and mRNA processing (de Souza et al., 2012). In the future, exploring the functions of these yet null TAL effectors in some processes rather than virulence by using the corresponding mutants will be an interesting research area.

Table 2-1. List of bacterial strains and plasmids used in the study

Strain or plasmid	Relevant characteristics	Source
Strains		
X. citri subsp. *citri*		
Xcc306	Group A, wild-type, Rif ^{r}	DPI[a]
Xcc306Δ*pthA1*	*pthA1* deletion mutant	This study
Xcc306Δ*pthA2*	*pthA2* deletion mutant	This study
Xcc306Δ*pthA3*	*pthA3* deletion mutant	This study
Xcc306Δ*pthA4*	*pthA4* deletion mutant	This study
Xcc306Δ*pthA1*Δ*pthA2*Δ*pthA3*	*pthA1, pthA2, pthA3* deletion mutant	This study
Xcc306Δ*4pthA*	*pthA1 pthA2, pthA3, pthA4* deletion mutant	This study
Xcc306Δ*pthA4*:PthA4	PthA4 complement Xcc306Δ*pthA4*, Gm^{r}	This study
Xcc306Δ*pthA4*:Pthw	PthAw complement Xcc306Δ*pthA4*, Gm^{r}	This study
Xcc306Δ*pthA4*:PthA*	PthA* complement Xcc306Δ*pthA4*, Gm^{r}	This study
Xcc306Δ*pthA4*:PthB	PthB complement Xcc306Δ*pthA4*, Gm^{r}	This study
Xcc306Δ*pthA4*:PthC	PthC complement Xcc306Δ*pthA4*, Gm^{r}	This study
Xcc306Δ*pthA4*:*dCsLOB*	Artificial TALE targeting *CsLOB1* complement Xcc306*4pthA*Δ, Tc^{r}	This study
Xcc306Δ*pthA4*:*dCsN3*	Artificial TALE targeting *CsN3-1* complement Xcc306Δ*pthA4*, Tetra^{r}	This study
Escherichia coli		
DH5α	F^{-}*recA*ϕ80d*lac*ZΔM15	BRL[b]
DH5αλPIR	Host for pOK1; Sp^{R}, oriR6K, K2 replicon	(Huguet et al., 1998)
Agrobacterium tumefaciens		
EHA105	Rif ^{r}, Cm^{r}	
LBA4404	Contain pAL4404 plasmid	
Plasmid		
pOK1	Suicide vector, SacB	(Huguet et al., 1998)
pRK2073	Sp^{r} Tra^{+}, helper plasmid	(Daniels et al., 1984)
pBluescript KS(+)	Phagemid, pUC derivative, Amp^{r}	Stratagene
pLARF3, pLARF6	*rlx*$^{+}$RK2 replicon, Tc^{r}	(Staskawicz et al., 1987)
pUFR053	repW, Mob^{+}, LacZα^{+}, Par^{+}, Gm^{r}	(El Yacoubi et al., 2007)
pBI101	Binary vector with uidA gene, Km^{r}	Clontech
pUC118/35S	pUC18 derivative with 35S, Amp^{r}	G. Moore
pCAMBIA2200	Binary vector, Cm^{r}	Cambia

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[b] Brl, Bethesda Research Laboratories, Gaithersburg, MD

Amp = ampicillin, Cm = chloramphenicol, Gm = gentamycin, Km = kanamycin, Sp = spectinomycin, Rif = rifamycin, Tc= Tetracycline

Table 2-2. List of primers name, the corresponding sequences and purposes

Primer	Sequence	Application
pthA1F	TGCCGCTTGCTGCAACAGAAG	Amplification of
pthA1R	TTGGCATCAGAGTGACGAACAC	*pthA1* gene including up-and down-stream
pthA2F	CGAGACCCTATACCGCGAG	Amplification of
pthA2R	CTGGACATACCAGACACTCCA	*pthA2* gene including up-and down-stream
pthA3F	GATCTGGCTGTCGGTAAAGCG	Amplification of
pthA3R	CCCTCACGCAAGCCGCTAT	*pthA3* gene including up-and down-stream
pthA4F	CACATAACGCGAGATTCCACG	Amplification of
pthA4R	TGCTTCAGTCCCTGATTGCC	*pthA4* gene including up-and down-stream
pthA4OEF	CCGCTCGAGCGGATGGATCCCATTCGTTCG	Amplification of
pthA4OER	GGAAGATCTTCCCTGAGGCAATAGCTCCATCA	*pthA4* gene for over-expression
37210F	TCCACCAACCGAACCATACA	Real-time PCR for
37210R	GGCACTTGCTTCATAGACCAT	*CsLOB1* gene
3027F	GTGAGCCTGAGAAACCATCG	Real-time PCR for
3027R	CCGTTGCCGTTAGCCATCT	*CsN3-1* gene
EF1aF	GTAACCAAGTCTGCTGCCAAG	Real-time PCR for
EF1aR	GACCCAAACACCCAACACATT	*CsEF1α* gene
37210PF	CCCAAGCTTGGGAACCTTGACCTGGAATGG	Amplification of
37210PR	CGCGGATCCGCGGCGTGGAGAAGATTGAGA	*CsLOB1* promoter
3027PF	CCCAAGCTTGGGTTGACGGACACCTCTTAA	Amplification of
3027PR	CGGGATCCCGTAGCATTTCCTGGCAACA	*CsN3-1* promoter
37210kpnF	GGGGTACCCCTTAACTTTGTTTCAACTAAAGC	Make EBE deleted
37210kpnR	GGGGTACCCCTATAGAGAAAGGAAAAGGC	*CsLOB1* promoter CsLOBPD
3027kpnF	GGGGTACCCCTTCTAGTCTGCTACCCACAA	Make EBE deleted
3027kpnR	GGGGTACCCCGGGAATTCAAAGAAACTAAC	*CsN3-1* promoter CsN3PD
37210xhoF	CCGCTCGAGCCTTAACTTTGTTTCAAC	Making EBE
37210xhoR	CCGCTCGAGGGGTTTATATAGAGAAAG	mutated *CsLOB1* promoter CsLOBPM1

Table 2-2. Continued.

Primer	Sequence	Application
3027xhoF	CCGCTCGAGCTTCTAGTCTGCTACCCA	Make EBE mutated *CsN3-1* promoter CsN3PM1
3027xhoR	CCGCTCGAGCGGTTTATATAGGGAATTC	
37210HindF	CCCAAGCTTGGGCTATATAAACCCCTTTTG	Making truncated *CsLOB1* promoter CsLOBPT
3027HindF	CCCAAGCTTCCTATATAAACCGCTTTTG	Making truncated *CsN3-1* promoter CsN3PT
LOBPmut	CCCAAGCTTGGGCTATATAAACCtCTTTTGCCT	Making EBE mutated *CsLOB1* promoter CsLOBPM2
LOBPmug	CCCAAGCTTGGGCTATATAAACCggTTTTGCCT	Making EBE mutated *CsLOB1* promoter CsLOBPM3
LOBPig	CCCAAGCTTGGGCTATATAAACCCCTTgTTGCCT	Making EBE mutated *CsLOB1* promoter CsLOBPins
37210M5F	TTCTCGAGATAAACCCCTTTTGC	Making 5’ EBE mutated *CsLOB1* promoter CsLOBPM5
37210M5R	TACTCGAGAAAGGAAAAGGCAAG	
37210OEF	ACGCGTCGACATGGAATGCAAACACAAAAT	Amplification of *CsLOB1* gene for over-expression
37210OER	CCGCTCGAGATCATGTCCACAGAGGCTC	
3027OEF	CGCGTCGAC ATGGATATTGCACATTTCTTG	Amplification of *CsN3-1* gene for over-expression
3027OER	CCGCTCGAGTCAAACTTGTTCAACTAGAGCC	
7877F	ACAGATTCAGCACAGAAGAGTT	Real-time PCR for Cit.7877.1.S1_at
7877R	GAAGCAAGGTCACCGTCAC	
2392F	CGTCAACCGTAAAAGCAGAA	Real-time PCR for Cit.2392.1.S1_at
2392R	GAGATGAACCCCTGTGATGAA	
5370F	CGTCCACAACAGCCAAATC	Real-time PCR for Cit.5370.1.S1_s_at
5370R	AGGCGTGCGATGAGAGATAC	
39387F	TGCTATTGGTGGAAGTGCTG	Real-time PCR for Cit.39387.1.S1_at
39387R	CACTCTCTGGTGCATCCTCA	

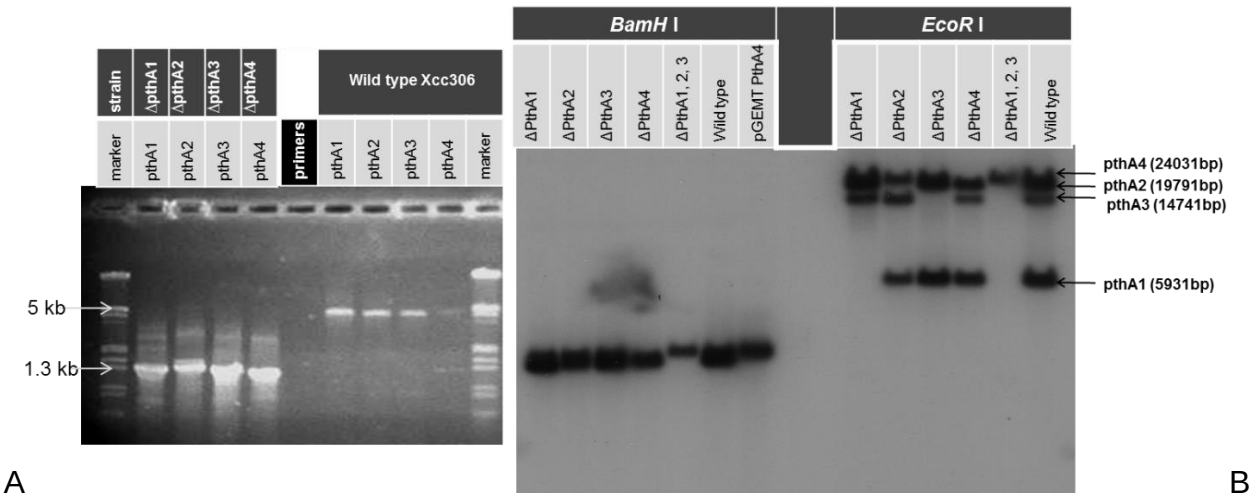


Figure 2-1. Confirmation of the mutants by PCR and Southern Blot. A) PCR amplification of the genome DNA of mutants Xcc306Δ*pthA1*, Xcc306Δ*pthA2*, Xcc306Δ*pthA3*, Xcc306Δ*pthA4* using the respective primers upstream and downstream of the genes, wild-type Xcc306 genome DNA was also amplified with these four primers. The marker indicates products size. B) Southern blot analysis of Xcc306 *pthA* deletion mutants. The genome DNA was digested with *BamH* I and *EcoR* I, and the picture was taken after 120 min of exposure.

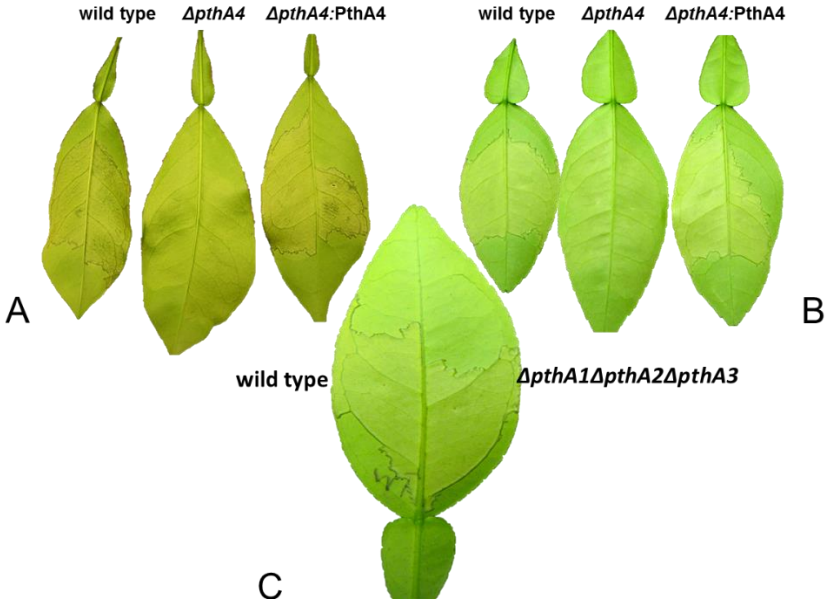


Figure 2-2. Knocking out of *pthA4* resulted in loss of pathogenicity in sweet orange and grapefruit. A) In sweet orange, the *pthA4* deletion mutant caused drastically reduced pustule formation (middle), and can be complemented back (right). B) In grapefruit, the same situation as in A. C) The deletion of *pthA1*, *pthA2* and *pthA3* in triple mutant Xcc306Δ*pthA1*Δ*pthA2*Δ*pthA3* (with *pthA4* intact) did not affect the pustule formation in grapefruit. The leaves were photographed 5 days after inoculation.

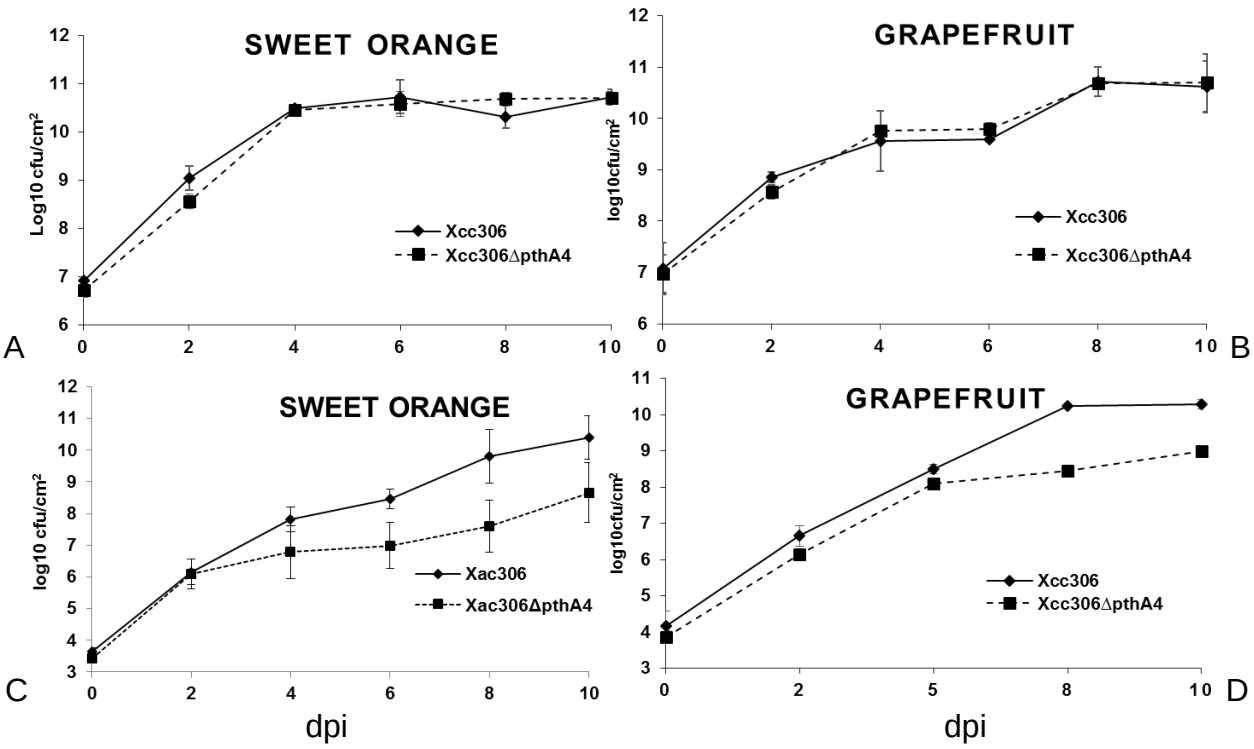


Figure 2-3. Bacterial growth curves in sweet orange and grapefruit for wild-type Xcc306 and mutant Xcc306Δ*pthA4*. A-B) No differences in bacterial leaf population when the inoculum concentration was 5×10^8 cfu/ml for sweet orange (A) and grapefruit (B). C-D) The leaves were infiltrated at concentration of 5×10^5 cfu/ml. In sweet orange, the bacterial growth of the mutant was much lower than that of wild-type from 4 dpi (days post inoculation) (C), while there was slight difference between them in grapefruit (D). The populations were monitored at the time points indicated. Error bars represent standard deviations (SD). The experiment was repeated twice with similar results.

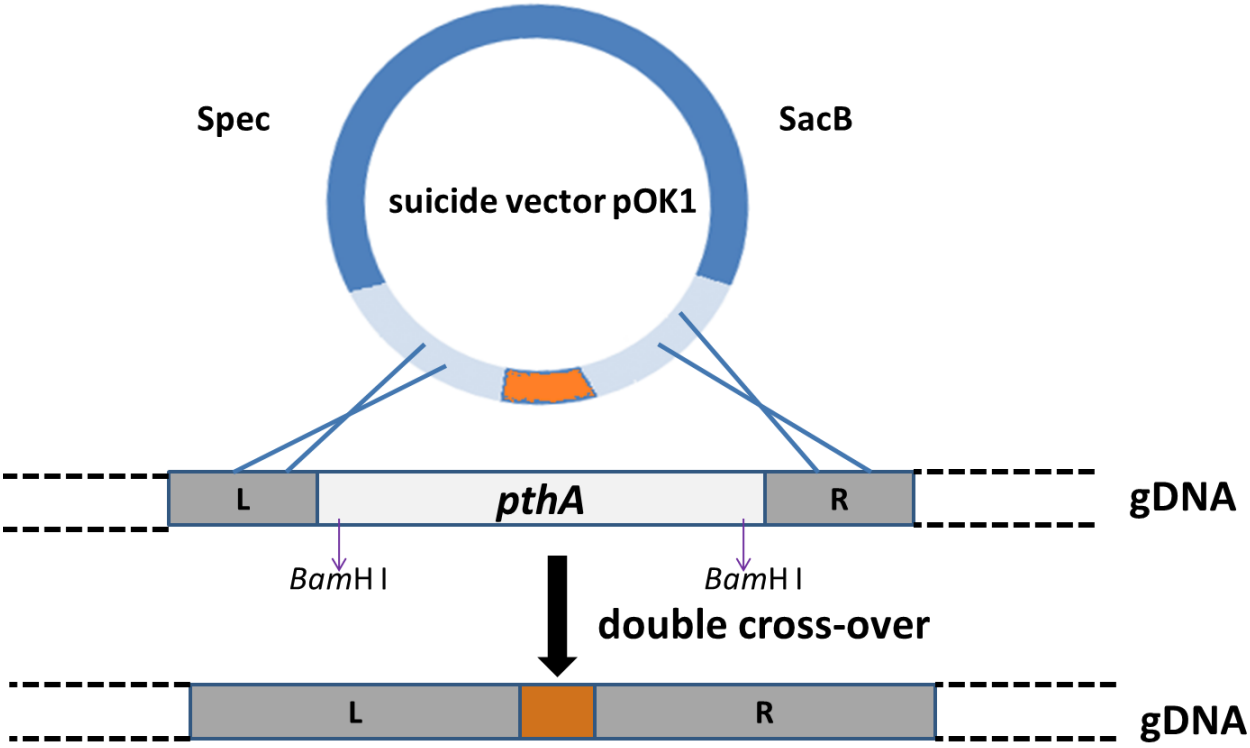


Figure 2-4. Mutagenesis process for *pthAs* gene deletion. The pthA genes including left (L) and right (R) flanking sequence were amplified and digested with *Bam*H I. The deleted fragment was transferred to pOKI harboring spectinomycin (Spec) resistance gene and sucrose sensitive gene (SacB). The mutant strains were generated by screening and double cross-over.

CHAPTER 3
TRANSCRIPTION PROFILLING OF SWEET ORANGE IN RESPONSE TO PTHA4-MEDIATED INFECTION

Background Information

After being attacked by pathogens, the expression of some plant host genes will be altered, probably as a result of defense or susceptibility interactions. Here, some previous work on the reprogramming of plant transcriptome triggered by pathogens is provided.

The Expression of Citrus Host Genes Responding to Xcc Infections

Strategies to study citrus plant responses to *Xanthomonas*. In comparison to the massive investigation of mechanisms employed by the bacterium relating to citrus canker disease development, with respect to the hosts, limited knowledge about plant host defense responses are available. An et al. (An and Mou, 2012) established a non-host pathosystem involving Arabidopsis and *Xanthomonas citri* subsp. *citri* (Xcc) in order to identify defense genes activated by Xcc; they demonstrated that the SA signaling pathway may play a critical role in resistance to Xcc. Although no specific gene in citrus that responds to Xcc infection has been well characterized yet, commercially availability of the citrus Genechip array (Martinez-Godoy et al., 2008) has resulted in several studies with high-throughput transcriptome profiling being conducted to exploit gene expression upon the invasion of Xcc or *X. fuscans* subsp. *aurantifolii* (Xfa). These results have helped to greatly expedite our efforts to understand the molecular mechanisms underlying pathogen infection processes and facilitate the elucidation of citrus defense response at the molecular level. A summary of these studies is discussed below.

Transcriptional analysis of sweet orange following challenged with compatible and incompatible *Xanthomonas*. Cernadas *et al*. (Cernadas et al., 2008) surveyed the early molecular events leading to canker development by performing transcriptional analysis of sweet orange plants infected with the pathogens Xcc306 and Xfa-C strain, which elicit canker disease and HR, respectively. By using suppressive subtractive hybridization and microarray analysis, they observed significant changes in the expression of defense, cell-wall, hormone, vesicle trafficking (such as syntaxins and PDR/ABC transporter etc.), transcriptional regulator and cell division genes in Xcc306-infected leaves relative to mock (water) inoculation, from 6 hpi to 48 hpi. Since Xfa-C elicits an incompatible reaction in sweet orange, it triggered a mitogen-activated protein kinase signaling pathway involving WRKY and ethylene-responsive transcriptional factors known to activate downstream defense genes. Furthermore, the genes commonly regulated by Xcc306 and Xfa-C were associated with basal defenses triggered by pathogen-associated molecular patterns, including those involved in reactive oxygen species production and lignification. Specially, they recognized a vesicle trafficking inhibitor, brefeldin A, which retarded canker symptom development.

Genome-wide molecular responses of citrus to *Xanthomonas* regarding resistance and susceptible. To unravel the molecular mechanisms underlying the Xcc resistance and susceptible, Fu and Liu (Fu et al., 2012; Fu and Liu, 2013) conducted two studies to investigate the global gene transcriptional changes by comparing several citrus varieties after Xcc infection. One involved the canker-resistant cultivar Meiwa kumquat (*Fortunella crassifolia*) and the second one involved a canker-resistant transgenic sweet orange produced via overexpressing a spermidine synthase, and the

third one is a canker-sensitive wild-type sweet orange cultivar. They showed that genes related to the cell wall and polysaccharide metabolism were induced in both canker-resistant and –sensitive species. Moreover, the expression of the genes involved in response to biotic stimulus, defense response, and cation binding was especially up-regulated in kumquat in comparison to sweet orange. Notably, abundant photosynthesis-related genes were significantly down-regulated in sweet orange. They also discovered that genes in categories of stimulus response, cell wall, transcription factors, starch and sucrose metabolism, glutathione metabolism, biosynthesis of phenylpropanoids, and plant hormones played major roles in canker resistance, when compared the gene expression between the infections of transgenic lines and wild-type lines. Another study conducted by Khalaf *et al*. (Khalaf et al., 2011) revealed comprehensive gene expression involved in the incompatible interaction following inoculation of kumquat with Xcc. In their survey, most of the differentially expressed genes involved in defense mechanisms in kumquat were associated with an HR and included oxidative burst, protein degradation, and regulation of photosynthesis as well as the production of ROS that is associated with the oxidative burst.

Transcriptional Responses of Citrus to Infection with Other Pathogenic Bacteria

Expression of citrus genes subsequent to non-host *Xanthomonas* inoculation.

Through inoculation of citrus with the non-host bacterium *X. campestris* pv. *vesicatoria* (Xcv), Daurelio *et al*. (Daurelio et al., 2013) identified 58 genes encoding transcription factors including some from the stress-associated families AP2-EREBP, bZIP, MYB and WRKY differentially regulated; 10 of the 58 TFs were specifically over-represented in citrus stress libraries, which may be important factors in citrus disease defense.

Transcriptional profiles of citrus in response to *Candidatus* Liberibacter asiaticus. The global gene expression of *Citrus sinensis* was also exploited following infection with the phloem-limited bacterial pathogen *Candidatus* Liberibacter asiaticus (Las) which causes Huanglongbing (Kim et al., 2009b; Albrecht and Bowman, 2012; Zheng and Zhao, 2013). Following inoculation, expression of genes associated with cell cycle, sugar metabolism, plant defense, phytohormone, photosynthesis, transcriptional regulation, transport and cell wall metabolism was observed to be affected by Las infection.

Bacterial Effectors-Mediated Gene Expressions in *planta*

Effector-based gene expression in Arabidopsis by infection of *Pseudomonas.* Microarray is a high-throughput and powerful technology for deciphering the molecular response under different treatments, and an explosion of studies has focued on plant global gene expressions as affected by compatible or incompatible host pathogen interactions through the comparisons of inoculation with pathogens and mocks; however, limited work had been done to examine plant genomic-wide transcriptional responses specifically to the bacterial effectors which mediating the subverting of basal defense responses. These studies will help to elucidate the genes involved in effector-mediated susceptibility (ETS) as well as ETI. Truman *et al*. (Truman et al., 2006) universally surveyed the genes specifically involved in type three effectors (TTEs) which orchestrated basal defense during compatible interactions in Arabidopsis, by comparing the invasion of *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) with the TTEs delivery deficiency strain DC3000 *hrp*$^{-}$. The differences in host gene expression between DC3000 and *hrp*$^{-}$ challenges revealed that the TTEs strikingly suppressed the endomembrane targeted, and more specifically, LRR receptor-like

kinase genes, pigment biosynthesis transcripts, genes involved in primary carbon metabolism in the plastid, genes associated with aromatic biosynthesis and phenylpropanoids, while MYB, NAC and AP2 transcription factor families were over-represented within the TTEs induced categories. In a proteomic study, Kaffarnik *et al*. (Kaffarnik et al., 2009) indicated that effector proteins of the pathogen *Pseudomonas syringae* may manipulate host secretion.

Induced, suppressed and targeted genes in plants by effectors in other examples. Marois *et al*. (Marois et al., 2002) conducted cDNA-AFLP analysis of pepper gene expression through the comparison of the infections with X. *campestris* pv. *vesicatoria* strain 85-10 expressing AvrBs3 or AvrBs3ΔAAD (ADD domain deletion mutant); 13 induced genes were identified, which included members of the auxin-induced gene family, α-expansin gene, pectate lyases and anthocyanidin rhamnosyl transferase genes. The expression of tomato genes was also monitored upon inoculation of isogenic *X. campestris* pv. *vesicatoria* (*Xcv*) strains differing only in the *avrXv3* gene which elicits a resistance response in tomato; 139 genes and 1,294 genes were observed to have significant changes in transcript levels at 8 h and 12 h post inoculation respectively (Balaji et al., 2007). In particular, multiple genes were characterized as being involved in effector-mediated susceptibility or resistance. The AvrBs3/PthA proteins are distinguished in inducing plant target genes (more details in next chapter). The transformation with *avrBs3/pthA* genes *avrXa7*, *avrXa10*, and *apl1* abolished the HR in tobacco leaves elicited by *Pseudomonas fluorescens*, resulting in the suppression of defense response related genes, which included *PAL* (Phenylalanine ammonia-lyase), *PR1*(pathogenicity-related 1), and *RbohB* (NADPH oxidase gene)

(Fujikawa et al., 2006). Two type III effectors from *X. campestris* pv. *vesicatoria* (*Xcv*), XopB and XopS, which contributed to increased disease symptoms and bacterial population, were demonstrated to suppress the basal defense response acting downstream or independent of MAPK activation (Schulze et al., 2012). In plants, an astonishing number of components in PTI associated with receptor kinase in plasma membrane (like FLS2, BAK1, RIN4 and PBS1), chloroplast, vesicle trafficking, MAPK signaling, and nucleus were targeted by effectors; nevertheless, most of them were identified through protein-protein interaction approaches, and were determined to be substrates of the effectors and key modules in response to effectors by forming a protein complex, although they were not necessarily affected in transcription level (Deslandes and Rivas, 2012; Feng and Zhou, 2012). However, the family of TAL effectors including PthA4 is an exception in that they directly target the promoter of host genes rather than the translated proteins, and they fortify the genes at the mRNA level.

Results

Microarray Analyses Overview

To gain insight into the genes involved in PthA4-based plant susceptibility, one of the sweet orange citrus varieties, Valencia, was inoculated with wild-type Xcc306 (WT) and the less aggressive mutant Xcc306Δ*pthA4* (MU). Infected leaf tissue was sampled at 6 hpi, 48 hpi and 120 hpi, and global transcriptome profiles were examined through microarray analysis upon comparing the infiltrated tissues of these near isogenic strains. The expression fold changes of expressed sequence tags (ESTs) in citrus genome GeneChip array from Affymetrix were evaluated, which contains 30,171 probesets representing up to 33,879 citrus transcripts. The annotations of those ESTs were obtained from HarvEST:Citrus software which was originally released by the

USDA/CSREES Plant Genome program; the sequences and annotation information of the probesets was also deposited in PLEXdb (http://www.plexdb.org/modules/PD_probeset/annotation.php?genechip=Citrus). The genes with expression fold change equal to or greater than 3.0 (log fold change of 1.58, adjusted *P* value ≤ 0.05) between the infection of Xcc306 and pustule-defective strain were considered as differentially expressed (DE) genes at a statistically significant level in this study. In this study, we concentrated on the DE genes between infection of WT and MU at 48 hpi (48 hpi WT versus MU), which are supposed to be a demonstration of molecular response to PthA4-mediated susceptibility.

Quantitative reverse transcription-PCR (qRT-PCR) was conducted to verify the reliability of the microarray data with randomly selected gene-specific primers. Through qRT-PCR analysis, the selected gene expression data at 48 hpi were in consistent with their expression in microarray to some extents as shown in Figure 3-1, which potentially validate the overall microarray results. At 6 hpi and 120 hpi, the DE genes were also recorded to observe the dynamics of PthA4-specific response. In addition, the genes that changed in mRNA level were further reported by comparing the gene expressions at different time points after WT or MU infiltration, which may reveal the molecular process of disease development. The DE genes were then assigned to different categories based on putative functions using three different programs, MapMan software (Thimm et al., 2004; Usadel et al., 2005), BLAST2GO software (Conesa et al., 2005) and the web-based Singular Enrichment Analysis (SEA) tool in AgriGO program (Du et al., 2010)

The microarray data showed that at 48 h after infiltration, a total of 1895 probesets behaved as DE genes, and accounted for 4.35% of the total transcripts in the citrus GeneChip. 1311 of those citrus genes were up-regulated following invasion of Xcc306 relative to that of Xcc306Δ*pthA4*, and they were regarded as genes induced by PthA4, while 584 genes were significantly suppressed by PthA4. Then they were functionally categorized according to their similarity to genes with known functions in plants.

Up-Regulated Genes at 48 hpi Based on WT versus MU Infection

MapMan analysis. Firstly, we found that the highest up-regulated genes were related to cell wall metabolism (pectate lyase, expansin, pectin methylesterase inhibitor, cellulase, polygalacturonase) through the blast of DE genes. MapMan Wilcoxon Rank Sum Test was employed to classify the DE genes and calculate whether the probability that the DE genes assigned to a BIN or sub-BIN (function category) was statistically different from all other BINs or sub-BINs (*P* value < 0.05), which gave an informative snapshot of citrus response to PthA4-mediated Xcc306 invasion. According to the test, the main significantly up-regulated categories were related to cell wall, DNA synthesis/chromatin structure and protein synthesis (Table 3-1). The PthA4 induced genes that related to cell wall mainly focus on cell wall degradation and modification; in the category of DNA synthesis, primarily the histone related genes were significantly induced. Moreover, the genes expressing ribosomal protein synthesis represented most of the up-regulated genes that are involved in protein synthesis. However, some of the categories, such as phenylpropanoids and brassinosteroid metabolism, and bZIP transcription factor family members, did not contain large numbers of up-regulated genes although the category was significantly induced (Table 3-1). Although less

significantly enriched, it is worth mentioning the presence of other bins that contained abundant and relatively high proportions up-regulated genes (have more than 10 elements**,** *P* value >0.05). The proportion of each annotated category is displayed in Figure 3-2**.** These categories include lipid metabolism, hormone metabolism, stress, nucleotide metabolism, miscellaneous enzyme families, transcription regulator, protein, signaling, cell, development and transport. The up-regulated genes in lipid metabolism were almost evenly distributed into the sub-BINs. The hormone metabolism category includes auxin, brassinosteroid, and gibberellin related genes. The activated genes in category of transcriptional regulator mainly include the transcription factors WRKY domain family, Basic Helix-Loop-Helix (bHLH) family, bZIP transcription factor family, SET-domain transcriptional regulator, and chromatin remodeling/assembly factors. In addition to protein synthesis, the induced genes that are involved in protein synthesis are also largely associated with posttranslational modification and ubiquitin degradation. The up-regulated genes in the signaling category were enriched in receptor kinases, calcium and G-protein pathways. However, the functions of a large number of up-regulated genes (40.8%) were unassigned, and included no reliable homology to genes deposited in public databases or unknown functions.

Classified by BLAST2GO software. To better understand the range of genes in response to PthA4-related invasion, Gene Ontology (GO) annotations using BLAST2GO software with tBLASTx (e-value 10^{-6}) were also performed to assign the DE genes to functional categories with respect to three Gene Ontology vocabularies, which included biological process, molecular function and cellular component. The software returned annotations for 519 sequences of the up-regulated genes (71.3%). The

distribution of the GO functional terms revealed that in the secondary level of biological process, cellular process and metabolic process were the most represented ones, and the other categories included primary metabolic process, regulation of cellular process, response to stress, DNA metabolic process, DNA replication, cellular biosynthetic process, oxidation-reduction process, gene expression, cell wall organization and so on. In term of molecular function, binding and catalytic activity possessed a higher proportion of up-regulated genes, which were further divided into categories of protein binding, ATP binding, DNA binding, cation binding, hydrolase activity, protein kinase activity, transferase activity and oxidoreductase activity. When the cellular component was concerned, most of the up-regulated genes were enriched in cytoplasm, intracellular organelle, plasma membrane, nucleus, cell periphery, cell wall and plastid (Figure 3-3).

KEGG annotation. The biological interpretation of the up-regulated genes that encoded enzymes was further performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation by BLAST2GO. It showed that the most prevalent pathways involved were (from the most to the least relative and more than 5 genes affiliated) starch and sucrose metabolism, pentose and glucuronate interconversions, pyrimidine metabolism, galactose metabolism, purine metabolism, and amino sugar and nucleotide sugar metabolism, of which some were in consistent with biological processes already shown by GO analyses.

Singular Enrichment Analysis (SEA) test. The enrichment analysis was also carried out by using the Singular Enrichment Analysis (SEA) tool in AgriGO program. Some of the GO terms were shown to be remarkably enriched of up-regulated genes

(FDR<0.05), with a higher percentage (input in the figure) than their overall percentage in the total EST (reference in the figure), which were defined as over-represented categories, and were more inclined to be up-regulated than other types (Figure 3-4). Based on biological processes, these categories mainly concentrate on DNA (protein-DNA complex assembly, DNA packaging, DNA replication), cell wall (cell wall organization and modification), and metabolism (carbohydrate, sucrose and starch metabolic process), while some other processes also include microtubule-based movement, protein polymerization and trichome differentiation. In contrast, at least two categories, regulation of transcription and catabolic process, are regarded as under-represented; they contain a lower percentage of up-regulated genes than their overall percentage. Concerning molecular function, the up-regulated genes were enriched in pectate lyase activity, pectinesterase activity, DNA-dependent ATPase activity, motor activity, copper ion binding and enzyme regulator activity. Among them, pectate lyase, pectinesterase and enzyme regulator activity are related to cell wall organization, while DNA-dependent ATPase activity and motor activity are in paralogy with DNA related process and microtubule-based movement respectively. Conversely, the transporter activity under-represented the up-regulated genes. When examining the cellular component, the up-regulated genes primarily over-represented in the categories of cell wall, endomembrane system, cytoplasmic vesicle, cytoskeleton, extracellular region, protein-DNA complex and membrane localization. The chloroplast part was under-represented. In conclusion, these over-represented categories are logically the ones that play essential roles in response to PthA4-dependent citrus canker.

Comparison of two categorization systems. Although distinct annotation systems were used (MapMan and gene ontology), some of the function categories were found to be involved in PthA4-mediated canker disease development by both programs, such as cell wall degradation and modification, DNA synthesis and chromatin structure, cell wall degradation enzymes and sugar metabolism.

Down-Regulated Genes at 48 hpi Based on WT versus MU

The PthA4-dependent down-regulated genes were also annotated and categorized using MapMan, BLAST2GO and AgriGO. Since these genes were suppressed in the susceptible reaction, we can reasonably consider most of them are involved in plant defense rather than disease development, or they are essential for the normal plant growth and development which were inhibited by the invasion of pathogens.

MapMan analysis. In MapMan Wilcoxon Rank Sum Test, the processes relating to photosynthesis, development, stress related PR proteins and receptor kinases signaling were the significantly down-regulated categories (Table 3-1). As expected, they were associated with plant resistance response and plant growth, which were restricted by pathogen attack. In addition, as was observed with up-regulated genes, several BINs contain a substantial number and portion of down-regulated genes although they were not significantly altered; these include lipid metabolism, amino acid metabolism, secondary metabolism, hormone metabolism, miscellaneous enzyme families, regulation of transcription, protein, minor CHO metabolism, and transport (Figure 3-5).

BLAST2GO analysis. In BLAST2GO analysis, 441 (75.5%) of the down-regulated genes were annotated and categorized into GO terms (Figure 3-6). In

biological process, the largest classes in the secondary categories included metabolic process, cellular process, and response to stimulus were; the higher level GO terms included oxidation-reduction process, cellular biosynthesis process, response to light, defense response, regulation of cellular process, and transport constituted the majority of down-regulated categories. In terms of molecular function, the largest sets of down-regulated genes were binding and catalytic activity, which were sorted into several major groups such as oxidoreductase activity, metal ion binding, electron carrier activity, endopeptidase inhibitor activity and others. In addition, for cellular component class, the majority of down-regulated genes code for proteins localized in cytoplasmic part, followed by intracellular membrane-bounded organelle, chloroplast and cell periphery.

KEGG pathway analyses. Furthermore, through KEGG pathway analyses, the down-regulated genes were predominately involved in pathways of flavonoid biosynthesis, phenylpropanoid biosynthesis, glycolysis / gluconeogenesis, phenylalanine metabolism, glycine, serine and threonine metabolism, tyrosine metabolism, tryptophan metabolism, isoquinoline alkaloid biosynthesis, galactose metabolism, alpha-Linolenic acid metabolism, phenylalanine, tyrosine and tryptophan biosynthesis and aminobenzoate degradation.

Singular Enrichment Analysis (SEA) interpretation. By using SEA tool in AgriGO, the significant down-regulated categories (FDR<0.05) were explored (Figure 3-7 and Table 3-2). With regard to biological process, it showed that the secondary metabolic process (include phenylpropanoid biosynthetic process), aromatic compound biosynthetic process (include L-phenylalanine metabolic process), organic acid biosynthetic process (include monocarboxylic acid metabolic process), amino acid

metabolism, lipid biosynthetic process, electron, peptide and carbohydrate transport were significantly repressed in PthA4-dependent Xcc306 infection. In molecular function, the over-represented categories were primarily catalytic activity (enzymes include oxidoreductase, monoxygenase, lyase, UDP-glycosyltransferase, glucosyltransferase), transport (especially sugar transmembrane transporter and symporter activity), binding (iron ion binding and heme binding) and enzyme inhibitor activity. With regard to cellular component, the down-regulated genes were significantly concentrated in cell wall, photosystem, thylakoid and organelle subcompartment. From these classifications, it indicates that the photosynthesis related categories were interpreted as being significantly enriched for down-regulated genes, which is consistent with the MapMan annotation. However, the significantly down-regulated metabolic process, transport and binding functions that appeared in AgriGo analysis were not shown to be significant in the MapMan test, although it is possible that overlaps exist between the functional categories in different annotation systems. On the other hand, the *P*-value calculation method and significance cutoff may also contribute to the divergence, since MapMan analysis did not classified several categories as significant altered ones, and contained lots of down-regulated genes.

Comparison of Down-Regulated and Up-Regulated Genes Distribution at 48 hpi WT versus MU

When comparing the categorization of up-regulated genes and down-regulated genes, it is noteworthy to extract the categories that contain sharply different number of these two type of genes. If a category contain much more up-regulated genes than down-regulated genes, we may come to the conclusion that this category is preferentially induced by PthA4-depedent Xcc306, and vice versa. This hypothesis will

help in more precisely interpreting the overall functions of up-regulated genes and down-regulated genes.

Comparison of the categories of up-regulated and down-regulated genes sorted by MapMan. First, several up-regulated genes were determined to participate in nucleotide metabolism, TCA, ATP synthesis, while none of the down-regulated genes were involved, and conversely, some vitamin metabolism associated genes were suppressed but none were induced (Figure 3-2 and 3-5). Intriguingly, some categories that were enriched for down-regulated genes contained only limited numbers of up-regulated items (such as photosynthesis, secondary metabolism, hormone metabolism, biotic stress, cytochrome P450, transport, glucosyl and glucoronyl transferase), while there were also categories with a large number of up-regulated genes that contained only few down-regulated elements (included cell wall, glucosidase and galactosidase, DNA, protein synthesis, nucleotide metabolism, G-protein signaling and cell) (Figure 3-8A).The gene with differentiated expressions and their corresponding categories were also displayed in MapMan visualized pathway diagram, with red being up-regulated and blue being down-regulated (Figure 3-8B), an alternative but more graphic and straightforward way to show the DE genes affiliations.

Distribution of Sub-BINs for down-regulated genes that are different from for up-regulated genes. Although the up-regulated and down-regulated genes were assigned to some common BINs, they were largely distributed in different sub-BINs. Most of the down-regulated genes involved in lipid metabolism process belong to lipid degradation related family; in amino acid metabolism, mainly the expression of amino acid synthesis related genes was inhibited; the secondary metabolism predominantly

included isoprenoids, phenylpropanoids and flavonoids metabolism; in hormone metabolism, ethylene and jasmonate associated genes occupied a large portion; cytochrome P450, UDP glucosyl and glucoronyl transferases were dominant in miscellaneous enzyme category; no transcription factor families was prevailing in the RNA group; in protein, lots of suppressed genes were associated with posttranslational modification and protein degradation; and in transport, the sugar, ABC, and peptides transporter categories contained more down-regulated genes than other sub-BINs. There were also 36.8% of the down-regulated genes with unassigned functions, which did not show any similarity to proteins present in public databases (Figure 3-5).

Comparison of the categories between up-regulated and down-regulated gene sets that are classified by BLAST2GO. The distributions of the BLAST2GO generated categories for up-regulated genes and down-regulated genes were compared (Figure 3-3 and 3-6). Intriguingly, while the distribution patterns of up- and down-regulated genes was very similar, obviously more proportion of up-regulated genes than down-regulated genes involved in cellular organization or biogenesis, development process, structural molecular activity and binding than that of, and conversely, metabolic process, response to stimulus, immune system, transporter activity, electron carrier activity, enzyme regulator activity, catalytic activity and membrane contained more percentage of down-regulated genes. Particularly, the categories of cell proliferation and membrane-enclosed lumen contained 35 up-regulated genes while no down-regulated genes was involved.

Characterization of DE Genes Following Inoculation of WT Relative to MU at 6 hpi and 120 hpi

Number of DE genes. The differentially expressed genes with comparison of WT versus MU at both 6 hpi and 120 hpi were annotated and classified by using MapMan and AgriGo-SEA. There were 29 genes and 913 genes up-regulated at 6 hpi and 120 hpi respectively when comparing the expression level of wild-type Xcc306 over mutant Xcc306Δ*pthA4* infection. In the meantime, 233 and 1094 down-regulated genes were observed at these two time points, respectively.

MapMan analysis. In MapMan study, when comparing the citrus gene expressions between the infection of wild-type and mutant, very few categories showed a significant difference at 6 hpi especially for up-regulated ones, while at 120 hpi, a large amount of genes as well as functional categories exhibited significant expression changes (Table 3-3). At 120 hpi, the significant up-regulated categories included cell wall degradation and modification, lipids synthesis, auxin response, redox regulation, development and transport. Meanwhile, the significant down-regulated categories were comprised of more categories including photosynthesis, cell wall, lipid metabolism, stress, miscellaneous enzymes, regulation of transcription, protein, minor CHO metabolism, light signaling, cell, glycolysis and TCA transformation. When compared categories that showed significant expression changes upon WT vs. MU infiltration between 48 hpi and 120 hpi, only the cell wall, photosynthesis and stress were shared in both time points. The distinct DE categories between these two time points may account for the disease development and plant physiological development, since the pustule formation symptoms are only visible after 4 days post infiltration.

Mercator analysis of the DE categories. The partitions of overall MapMan categories for the DE genes at 6 hpi and 120 hpi were carried out, considering the small number of genes up-regulated at 6 hpi; only the genes down-regulated at this time were sorted. At 6 hpi, when excluding the unassigned genes (39.5%), the highest percentage of down-regulated genes were in the categories of transport (half ABC transporter), stress (biotic), signaling (primarily receptor kinase), miscellaneous enzymes, RNA regulator and secondary metabolism (mainly Phenylpropanoids) (Figure 3-9A). At 120 hpi, 43.5% of the up-regulated genes and 38.6% of down-regulated genes were not assigned to any of the categories. In respect to the percentages of each functional category, they were compared between up-regulated and down-regulated data sets (Figure 3-9B and C). We noticed that cell wall, cell and DNA categories contained a large portion of up-regulated genes while it included few down-regulated genes; in contrast, the proportions of photosynthesis and secondary metabolism were high in down-regulated categories while they were drastically low in up-regulated ones.

Singular Enrichment Analysis (SEA) study. In AgriGO-SEA analysis, the categories that significantly enriched for DE genes were largely different when compared 6 hpi with that of 48 hpi and 120 hpi in respect to WT versus MU. But multiple GO categories were significantly affected by PthA4 simultaneously at 48 hpi and 120 hpi (Table 3-2). After 6 h of inoculation, no category was found to be significantly up-regulated by PthA4. The defense related (such as innate immune response, defense response, programmed cell death, ubiquitin ligase activity), carbohydrate binding and FAD binding categories were found to be over-represented in the down-regulated genes only at 6 hpi, which indicated that the Xcc306 secreted effector repressed the plant

basal defense at an early time of the infection. The over-represented categories were then compared between 6 hpi, 48 hpi and 120 hpi (Table 3-2). The down-regulated genes were significantly enriched in carboxylic acid biosynthetic process and heterocycle biosynthetic process at both 6 hpi and 48 hpi, but not at 120 hpi, while enriched in carboxylesterase activity only at 6 hpi and 120 hpi. Moreover, oxidoreductase activity and lyase activity categories were significantly down-regulated at all three time points. Apart from these two classes, most of the DE genes had very close distribution patterns at 48 hpi and 120 hpi. Uniquely, the categories of protein polymerization, cell wall modification, pectate lyase activity, chromosome, amino acid derivative biosynthetic, L-phenylalanine metabolic process, electron transport, carbohydrate transport, peptidase inhibitor activity, UDP-glycosyltransferase activity only over-represented the DE genes at 48 hpi, while regulation of cell cycle, lipid localization, cell division, nuclear division, response to gibberellin stimulus, hydrolase activity on glycosyl compounds, heterocycle catabolic process, protein-chromophore linkage, transferase activity, transferring glycosyl groups and chloroplast categories specifically enriched for DE genes at 120 hpi.

Commonly Differentially Expressed genes at three time point. Then the DE genes at 6 hpi, 48 hpi and 120 hpi were also used to investigate for their overlapping through Venn diagram analysis. No gene was found to be up-regulated by PthA4-based Xcc306 infection in all three of the time points. Interestingly, however, 27 genes were down-regulated at 6 hpi while up-regulated at both 48 hpi and 120 hpi, and had no explicit distributions to functional categories (Figure 3-10A). Moreover, only 1 gene, Cit.57.1.S1_x_at, which encodes a protease inhibitor protein was identified to be down-

regulated at all three time points, (Figure 3-10B). Unexpectedly, the amount and percentage of genes that were commonly regulated at 48 hpi and 120 hpi were not large, which may be due to a large number of genes involved in late disease development. These data indicated that gene expression in citrus displayed noticeable differences towards early stage (6 hpi) and late stage (48 or 120 hpi) infection.

Reprogramming of Gene Expressions between Different Time Post Inoculations

For the reason that diversity of the DE genes at 6 hpi, 48 hpi and 120 hpi, the genome-wide gene expressions in mRNA level were also compared between 6, 48 or 120 hpi for both wild-type Xcc306 and mutant Xcc306Δ*pthA4*.

The overlapping DE genes between WT and MU when compared 48 hpi and 6 hpi. The number of genes commonly changed in expression between distinct comparisons was shown in Venn diagram (Figure 3-11). It revealed that 364 up-regulated probesets and 399 down-regulated probesets overlapped between wild-type Xcc306 and mutant Xcc306Δ*pthA4* regarding 48 hpi and 6 hpi, and were considered to be regulated by time or the environment rather than by PthA4 (Figure 3-11A).

Categorization of DE genes at 48 hpi relative to 6 hpi infected by MU. After the inoculation of Xcc306Δ*pthA4*, 964 probesets were triggered and 902 were suppressed in relation to 48 hpi versus 6 hpi (fold change>3, adjusted *P* value <0.05). Following MapMan software test, 618 of the up-regulated probesets were assigned functions (Table 3-4). The categories of lipid metabolism, lignin biosynthesis, auxin responsive, biotic stress, heat stress, cytochrome P450, nitrile lyases, MYB-related transcription factor family, protein (mainly posttranslational modification and E3 ubiquitin), cell division and development were significantly induced (*P* value <0.05). Other categories that contained most up-regulated genes (more than 10 with p value >

0.5) also included photosynthesis, amino acid metabolism, secondary metabolism (predominantly isoprenoids and phenylpropanoids), hormone metabolism (chiefly split into ABA, auxin and ethylene), redox regulation, UDP glucosyl and glucoronyl transferases, transcription factor, protein degradation, minor CHO metabolism, signaling, cell organism and transport. Meanwhile, the significantly reduced expression categories at 48 hpi relative to 6 hpi primarily included cell wall, lipid transfer proteins, chalcones metabolism, auxin responsive, thiamine metabolism, PR-proteins, drought/salt stress, redox regulation, DNA and protein, while the gene repression was furthermore substantially found in the categories such as lipid metabolism, amino acid metabolism (mainly amino acid synthesis), secondary metabolism (phenylpropanoids, flavonoide *et al*.), hormone metabolism (mainly auxin and ethylene), stress, cytochrome P450, GDSL-motif lipase, nitrile lyases, transcription factor (MYB domain family, Basic Helix-Loop-Helix family *et al*.), protein (postranslational modification, protein degradation),signaling (receptor kinases), cell (cell cycle and cell division), development and transport. Some of the categories were shown in a contrasting visualized diagram (Figure 3-12A). The red blocks represent up-regulated while the blue ones represent down-regulated, with the number of squares reflecting the number of genes. We can easily recognize that the categories of receptor kinase, G-protein, cell wall, Flavonoids, peroxidase, phosphatases and ethylene contained much more down-regulated genes, while more up-regulated genes were associated with UDP Glycosyltransferases, cell division, dehydrogenase and ABA.

Categorization of DE genes at 48 hpi relative to 6 hpi by WT. The wild-type Xcc306 up-regulated 1147 probes while 1119 probes were repressed at 48 hpi when

compared to 6 hpi. Through the MapMan Wilcoxon Rank Sum analysis, the categories of cell wall degradation and modification, biotic stress, heat stress, nitrile lyase, MYB transcription factors, C2C2-like zinc finger family, DNA synthesis, ribosomal protein synthesis, signaling, G-protein and transport were significantly up-regulated, while the cell wall modification, lipid transfer proteins, isoprenoids metabolism, thiamine metabolism, biotic stress (mainly PR-protein), peroxidases, WRKY transcription factor family, RNA binding and light signaling were significantly down-regulated (Table 3-4). Among them, cell wall degradation, cell wall modification, DNA synthesis and ribosomal protein synthesis were also significantly up-regulated while PR-protein related biotic stress was significantly down-regulated in scenario of 48 hpi WT versus MU. The differentially expressed genes were also contrastingly and visually shown in the MapMan Pathway diagram **(**Figure 3-12B**).** Remarkably more down-regulated genes were involved in cytochrome P450, terpenes, flavonoids, phenylproanoids, peroxidase, ethylene and JA than that of up-regulated genes; among them categories of peroxidase, ethylene were in line with what displayed in MU 48 hpi versus 6 hpi (Figure 3-12A). The categories containing extraordinarily more up-regulated genes than down-regulated genes included classes of calcium, G-protein, cell wall, cell cycle, cell division and auxins.

Singular Enrichment Analysis (SEA) study. Furthermore, the AgriGO-SEA study was performed to interpret these time-based comparisons (Table 3-5). After this analysis, we primarily focused on the comparisons of WT 48 hpi versus 6 hpi and 48 hpi WT versus MU. It illustrated that the significantly up-regulated categories in both comparisons included starch metabolic process, sucrose metabolic process, DNA

packaging, protein-DNA complex assembly, DNA replication, plant-type cell wall organization, DNA-dependent ATPase activity, copper ion binding, the localization in cell wall, membrane, cytoplasm vesicle, extracellular region, cytoskeleton and protein-DNA complex, while the common significantly down-regulated categories included aromatic compound biosynthesis, lipid metabolic, electron transport, monooxygenase activity, oxidoreductase activity, peptidase inhibitor activity and heme binding.

Characteristic of DE genes when comparing 120 hpi and 48 hpi. The comparative analysis between 48 hpi and 120 hpi was conducted. At the first glance, the number of DE genes (both up-regulated and down-regulated) by both WT and MU with respect to this comparison was much smaller than 48 hpi versus 6 hpi comparison. The expression differentiation with infection of Xcc306 wild-type between 48 hpi and 120 hpi was supposed to reflect both host and disease developments, which may be reinforced by the observation that categories of cell (cell cycle and division) and development were significantly up-regulated as well as the significant down-regulation of cell wall and lipid metabolism.

Singular Enrichment Analysis (SEA) test of DE genes in comparison of 120 hpi and 48 hpi. Based on AgriGO-SEA study, following wild-type Xcc306 infiltration, the categories of cell, development, 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, heme binding were significantly up-regulated at 120 hpi when comparing with that at 48 hpi, and the main significantly down-regulated categories included cell wall, lipid metabolism, biotic stress, oxidases, glucan metabolic

process, lipid and peptide transport, response to auxin stimulus, developmental growth, monooxygenase activity and carboxylesterase activity (Table 3-5).

Materials and Methods

Microarray Experiment and Analyses

Xcc306 wild-type strain and Xcc306*ΔpthA4* mutant were used to inoculate sweet orange (*Citrus sinensis*) at the concentration of 5 X 10^8 cfu/ml using needle with syringe in a quarantine greenhouse facility at the Citrus Research and Education Center, Lake Alfred, FL. The leaves were harvested 6, 48, 120 hours after inoculation for RNA isolation. To allow stringent statistical data analysis, three biological replicates were conducted and three technical replicates were pooled for each strain per time-point. RNA extraction was performed by using RNeasy Plant Mini Kit (Qiagen, Valencia, CA), the quantity and quality of RNA were determined on a ND-8000 Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington,DE). Microarray was conducted using Affymetrix array containing 33,000 Citrus spp. genes which is commercially available, labeling, hybridization, washing, scanning, and data analysis were performed at ICBR facility at University of Florida in Gainesville. Statistical tests were performed using BioConductor statistical software, open source software based on the R programming language (http://www.bioconductor.org/). Robust Multichip Analysis (RMA) approach was used for normalizing the raw data. Differential expression analysis was carried out using a linear modeling approach and the empirical Bayes statistics as implemented in the Limma package. Differentially expressed genes only with an adjusted *P*-value (FDR) less than 0.05 were considered as differentially expressed genes at a statistically significant level.

Quantitative Reverse Transcription-PCR Analyses

The leaves were sampled 48 h after syringe-infiltration (5 X 10^8 cfu/ml), the total RNA was extracted by using TRIzol Reagent (Ambion, Carlsbab, CA) following the manufacturer's instruction. 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, Gaitherburg, MD, USA). The gene-specific primer sequences are listed in Table 3-2. The elongation factor gene *EF1α* was used as endogenous control. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification.

Categorization of DE Genes

For MapMan analysis, the microarray data of each comparison with log2 fold change value and FDR value was loaded into the software, a filter with FDR<0.05 was set, the pathways were visualized in the context of built-in Citrus_AFFY mapping. The data with only DE genes the corresponding log2 fold change value was also loaded, 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 significantly different regulation compared to all other BINs by *P*-value calculation.

Gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation were performed to classify the functions of DE genes using Blast2GO software. The high-throughput sequences of the DE genes were gotten from Get Sequence tool in PLEXdb basing on Affymetrix IDs (http://www.plexdb.org/modules/tools/get_seq.php). The putative functions were assigned through blasting, mapping annotation and InterProScan functional labeling. GO terms for each of the three main group (biological process, molecular function, and

cellular component) were obtained by using the combined graphs function of the software with default parameters. The KEGG analysis were performed by using the KEGG annotating function of the program. To analyze GO term enrichment of DE genes, SEA was performed online through agriGO program, a GO analysis tool kit for the agricultural community (http://bioinfo.cau.edu.cn/agriGO). Briefly, the DE gene probe IDs were first uploaded into the agriGO, and the Citrus Affymetrix Genome Array was selected as the reference, statistical *P*-values were calculated using the Fisher method, and multiple-test adjustment using the Yekutieli (FDR under dependency) method. The Venn diagrams were generated by using an online program Draw Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Discussion

In this chapter, the reprograming of transcriptional profiles in sweet orange was scrutinized after the infiltration of wild-type Xcc306 and a *pthA4* deletion mutant at three time point. Despite the entire *Citrus Sinensis* transcriptome cannot be fully represented by the citrus transcripts on the array, and the possibility of post-translation modification as well as technical and statistical issues, the microarray with the GeneChip is still the most effective tool to explore the citrus global gene expression, although it is only a descriptive rather than a validation approach. Without the presence of effector PthA4, the bacterial strain Xcc306Δ*pthA4* was not able to conquer the plant basal defense response. Here, I focused on the outstanding DE genes and functional categories that were affected by infection of Xcc306 at 48 hpi relative to both infection of Xcc306Δ*pthA4* at 48 hpi and infection of Xcc306 at 6 hpi. These two comparisons were considered to reflect the molecular response of plant vulnerability.

Cell Wall Associated Genes

According to the MapMan function categorization, 43 cell wall-related genes were significantly up-regulated in 48 hpi WT versus MU (Table 3-1), which is the BIN containing the largest number of up-regulated elements. Plant cell wall is the first physical barrier for invasion by bacterial pathogens, in which the bacteria employ multiple strategies to break and traverse the cell wall. Most of the strategies are through enzymatical activity, either by secreting cell-wall-degrading proteins (CWDPs) or promoting plant cell wall remodeling enzyme genes expression. The plant cell wall appositions papillae, which are composed of callose, phenolics (such as lignin), hydroxyproline-rich glycoproteins (e.g. expansins), cell wall polymers including pectin and xyloglucans (Underwood, 2012). The plants CWDPs which include polygalacturonases (PGs), pectate lyases (PELs), pectin acetylesterase (PAE), expansin etc., can modify the composition and structure of wall polysaccharides, causing the disassembly of the cell wall and the increase of susceptibility, while cellulose synthase, xyloglucan-specific endoglucanase inhibitor protein (XEGIP) and pectin methylesterase inhibitor (PMEI) might enhance disease resistance (Juge, 2006; Cantu et al., 2008; Enrique et al., 2011). RNAi silencing of a callose synthase gene, *CalS1*, made citrus plant more susceptible to Xcc306 (Enrique et al., 2011). In another aspect, chitinase, endo-β-1, 4-glucanase and β-1, 3-glucanase, which are involved in the degradation of pathogen cell wall major components such as chitin, glucan, β-glucosidases and peroxidase (Escamilla-Trevino et al., 2006), can inhibit the invasion of bacterial and improve plant immunity (Wan et al., 2008). According to the functional categories of the up-regulated genes at 48 hpi and 120 hpi in WT vs. MU as well as that in WT 48 hpi vs. 6 hpi (Table 3-1-4) most of the cell-wall related genes are associated

with cell wall disassembly components like pectate lyase, cellulase, expansin, endoxyloglucan transferase, PME. Moreover, in the class of enzymes (direct to KEGG and Miscellaneous enzymes family), we also found that high quantity of the up-regulated genes expressed cell-wall degradation related enzyme. As indicated above, a large amount of the highest up-regulated genes are cell wall degradation related, which indicats that overcoming the plant cell wall is very crucial for the establishments of pathogen colonization. In another aspect, it also indicates that PthA4 may direct the disorganization of plant cell by target the genes which act as cell wall metabolism regulator.

Cytochrome P450 Family

Plant cytochrome P450 (CYP) is a group of heme-containing monooxygenases, which was reported to play paramount roles in the synthesis of plant defense compounds that served as toxic inhibitors against bacterial and fungal, such as monoterpene indole alkaloids that inhibit microtubule formation and cell division, and DIMBOA that inhibits protease and oxidative enzymes in bacteria or fungi (Schuler and Werck-Reichhart, 2003). Twelve hours post invasion of *Xanthomonas arboricola* pv. *pruni*, the down-regulation of CYP genes was the most pronounced transcriptional change in peach leaves (Socquet-Juglard et al., 2013). CYP genes in pepper and citrus were also found to be involved in defense responses against microbial pathogens in transcriptome analysis (Khalaf et al., 2007; Hwang and Hwang, 2010; Fu et al., 2012). In this study, the CYP and monooxygenase activities were remarkably suppressed by the Xcc306 when either compared to *pthA4* deficiency mutant or compared to earlier response (Table 3-2, 2-5, Figure 3-8, 2-7). In the comparison between WT 48 hpi and 6 hpi, 32 genes encoding cytochrome P450 were down-regulated, while 25 and 35 CYP

genes were down-regulated at 48 hpi and 120 hpi respectively in WT vs. MU. Furthermore, from the GO enrichment analysis, monooxygenase activity was significantly repressed in all these three comparisons that associated with susceptible response, and the KEGG pathway analyses also showed that a large amount of monooxygenases involved metabolic pathways were down-regulated. Interestingly, in the comparison of MU 48 hpi against 6 hpi, which was the possible indicator of defense response, the cytochrome P450 category was also significantly up-regulated (Table 3-4).

Transcriptional Regulator

Another important category of the DE genes is the transcription factor family, which has always been widely reported to play significant roles in plant susceptibility and defense. The transcription factors are always associated with hormone signal transduction; for example, AP2/ERF transcription factors are associated with JA and ET signal transduction while SA signal transduction involves mostly WRKY and bZIP groups (Van Verk et al., 2009).

AP2/ERF transcription factors. In this study, most of the DE genes coding AP2/ERF domain transcription factors were repressed in the susceptible reactions, such as in WT 48 hpi vs. 6 hpi, 48 hpi WT vs. MU 48 and in 120 hpi WT vs. MU 120 hpi, 7, 3 and 4 of the down-regulated genes belonged to AP2/ERF transcription factor in these comparisons respectively, which was also one of the transcription factor categories that contain largest number of down-regulated genes, indicating that it may be associated with the plant basal defense. AP2/ERF genes are members of ethylene response factor (ERF) subgroup of AP2 transcription factor family that specifically bind with GCC box. It was also previously suggested that AP2/ERF transcription factor family played

important roles in disease resistance by involving several hormone signaling transductions (such as ethylene, jasmonic acid and salicylic acid) and coordinately integrating these pathways (Gutterson and Reuber, 2004; Xu et al., 2011). Overexpression of the genes encoding AP2/ERF type transcription factor increase tolerances against pathogen attack in tobacco (Park et al., 2001; Zhang et al., 2009).

MYB domain and related transcription factors. From this study, MYB related transcription factor genes are either up-regulated or down-regulated, in 48 hpi vs. 6 hpi by both WT and MU, which may demonstrated that they involved plant development and ETI. In Arabidopsis, MYB transcription factors are also key agents in regulatory networks controlling responses to pathogen attack, which may be attributed to their regulation of defence-related primary and secondary metabolites (like flavonoid) (Dubos et al., 2010). AtMYB30 is an activator of HR in response against bacterial pathogens through the regulation of very-long-chain fatty acid synthesis (Raffaele et al., 2008). AtMYB96 induced disease resistance response by promoting SA biosynthesis (Seo and Park, 2010).

WRKY domain transcription factors. The WRKY TFs are one of the most notable families implicated in coping with pathogen attack. They act in a complex defense response network as both positive and negative regulators, and are involved in both PTI and ETI (Eulgem and Somssich, 2007; Rushton et al., 2010). AtWRKY52 works as a recessive R protein conferring resistance toward the bacterial wilt *Ralstonia solanacearum* (Deslandes et al., 2002); AtWRKY70 acts at a convergence between SA- and JA-dependent defense pathways to render both basal and *R*-gene mediated defense (Li et al., 2006; Knoth et al., 2007); AtWRKY72 contributes to basal immunity

as well as gene-for-gene resistance (Bhattarai et al., 2010); AtWRKY7 and AtWRKY11/AtWRKY17 contributed negatively to basal resistance toward a *P. syringae* virulent strain (Journot-Catalino et al., 2006; Kim et al., 2006). In other plant species, the *WRKY* genes also have vital functions in response to pathogens (Pandey and Somssich, 2009). Particularly, in citrus, *WRKY* genes were the largest class of transcription factors differentially expressed in non-host responses elicited by *X. campestris* pv. *vesicatoria* (Daurelio et al., 2013). In this study, the DE genes that encode WRKY TFs are minor; however 4 *wrky* genes were induced at 120 hpi with respect to WT vs. MU. Three of them were also induced at 48 hpi vs. 6 hpi by WT, which may work as positive regulator for ETS while negative for defense in late stages. Meanwhile, five totally different *wrky* genes were inhibited by WT considering 48 hpi vs. 6 hpi but not in WT vs. MU; they probably played constructive roles in early plant defense responses or negative roles in ETI triggered by other effectors.

Family of bHLH transcription factors. The MYC family of transcription factors consists of basic helix-loop-helix (bHLH) proteins, although they mainly function in control of anthocyanin biosynthesis, plant cell proliferation, phytochrome signaling. They are also found to be involved in defense against pathogens (Kazan and Manners, 2013). AtMYC2 negatively regulates the expression of defense-related genes through JA pathways (Lorenzo et al., 2004), and it is required for the attenuation of Microbe-Associated Molecular Patterns (MAMPs)-activated defenses triggered by Flg22 (Millet et al., 2010). In pepper, a cell size regulator and hypertrophy contributor, *upa20*, which is induced and targeted by effector AvrBs3, encodes a bHLH transcription factor (Kay et al., 2007). Here, the expressions of three genes and five genes (two shared) encoding

bHLH transcription factor was promoted in the comparisons of 48 hpi WT versus MU and WT 48 hpi versus 6 hpi respectively, which demonstrated that this group of genes likely acting as positive regulator in PthA4-mediated susceptibility in citrus.

Other factors in regulation of transcription. Apart from the transcription factors discussed above, the basic leucine zipper domain (bZIP) transcription factor family also serves as positive or negative regulator in plant-pathogen interaction responses (Alves et al., 2013). In this study, two *bZIP* family genes were induced in 48 hpi WT versus MU and WT 48 hpi versus 6 hpi respectively, while five of this type were induced in MU 48 hpi versus 6 hpi. In addition, the regulations at the chromosomal level also participated in plant defense. The chromatin modification always happened at the *PR-1* and *PDF1.2* promoter, which in turn changed the plant defense status. This indicates that chromatin structure plays important roles in pathogen responses (Van Verk et al., 2009). Two probesets, Cit.6327.1.s1_at and Cit.23227.1.s1_at, which are involved in chromatin remodeling, were activated in both 48 hpi WT versus MU and WT 48 hpi versus 6 hpi, indicating their roles in stimulating plant susceptibility.

Plant Hormone Metabolism and Signal Transduction

Plant hormones play crucial roles in plants responding to a wide range of biotic stresses (Bari and Jones, 2009; Robert-Seilaniantz et al., 2011; Meldau et al., 2012). In this study, the up-regulated genes in comparisons of WT versus MU and 48 hpi versus 6 hpi by WT in hormone metabolism are mainly related to auxin and gibberellin (GA), while the down-regulated genes associated with hormone were primarily assigned to ethylene and jasmonate. Overall, the number and percent of the down-regulated genes involved in hormone metabolism were more than those of up-regulated genes (Figure 3-2, 3-5, and 3-8A).

Auxin. It has been demonstrated that auxin is involved in the attenuation of defence responses in plants, and the pathogen infections cause expression changes of the auxin signaling genes (Bari and Jones, 2009). It was revealed that abundant auxin-related genes were repressed in SA-mediated systemic acquired resistance (SAR), which included auxin-responsive *SAUR* (small auxin up RNA) genes and *Aux/IAA*-family genes; the exogenous auxin application promoted the development of *Pst* DC3000 disease development and pathogen growth, which proved that auxin might down-regulate host defence responses (Wang et al., 2007). It was also shown that blocking of auxin signaling restricted *P. syringae* growth and enhanced bacterial disease resistance, which implicated that auxin plays a role in disease susceptibility (Navarro et al., 2006). In pepper, five AvrBs3-induced genes were identified to be homologous to members of a family of auxin-induced genes, the *SAUR* family, which may play a role in cell enlargement (Marois et al., 2002). In peach leaves, two genes belonging to the auxin transport family and SAUR-like auxin-responsive family were suppressed at 12 hpi following the challenge by the invasive pathogen *X. arboricola* pv. *pruni* (Socquet-Juglard et al., 2013). In citrus, a great number of *Aux/IAA* related genes were similarly down-regulated by both compatible strain Xcc306 and incompatible strain Xfa-C (Cernadas et al., 2008). In our study, the auxin metabolism or auxin responsive genes were also shown to promote plant susceptibility, given that the number of up-regulated genes greatly exceed down-regulated genes in this group in both 48 hpi WT versus MU and WT 48 hpi versus 6 hpi (Figure 3-12B).

Gibberellin. There is also evidence suggesting that GA signaling components play critical roles in plant disease susceptibility or resistance. By stimulating degradation

of the growth repressor DELLA protein, GA leads to increased SA promoting resistance to biotrophs and susceptibility to nectrotrophs pathogens through the modulation of reactive oxygen species (ROS) levels in plants, since the exogenous usage of GA resulted in enhanced resistance to *Pst* DC3000 and susceptibility to *A. brassicicola* in Arabidopsis (Achard et al., 2006). However, the mechanism how GA functions in the defence or susceptibility responses to pathogen is largely unknown. Gibberellic acid-stimulated Arabidopsis (GASA) homologues, involved in GA biosynthesis and cell organization, were strongly induced by Xcc306 and Las in both this study and previous studies (Cernadas et al., 2008; Albrecht and Bowman, 2012). The expression of various citrus cell remodeling enzyme genes, including cellulases, pectinesterases, expansin and galactosyltransferase that are induced in Xcc306 infection were rapidly and commonly affected by auxin and gibberellin. Interestingly, exogenous addition of auxin to infected plants promoted citrus canker development whereas an inhibitor of gibberellin synthesis significantly hindered the auxin-induced transcription and the appearance of canker lesions, demonstrating that crosstalk may control citrus plant cell division and expansion triggered by Xcc (Cernadas and Benedetti, 2009). Four GA related genes were up-regulated in 48 hpi WT versus MU and WT 48 hpi versus 6 hpi, while only one was down-regulated in the scenario (Figure 3-8B). This demonstrated that GA associated genes preferentially attenuated disease defense response.

Ethylene and Jasmonate. Ethylene (ET) is a principal modulator in relation to a plants reaction to microbial pathogen attack. ET biosynthesis is activated by challenge with pathogens and in turn induces antimicrobial related genes as well as defence-related genes through a cascade of signaling pathways including AP2/ERF and

EIN3/EIL-type transcription factors, suggesting that the role of ET (synthesis and signaling) might be more associated with disease resistance than susceptibility in host-pathogen interactions (Broekaert et al., 2006). ET biosynthesis and perception promote defense response in citrus fruit against the fungus *Penicillium digitatum* infection (Marcos et al., 2005). Jasmonates (JA) modulate defense against necrotrophic pathogens through the coronatine insensitive 1 (COI1) F-box protein (Sheard et al., 2010). The concentration of JA was shown to increase in pathogen infection sites (Lorenzo and Solano, 2005). From Figure 3-8B and Figure 3-12B, we can clearly observe the expression patterns of ET and JA were converse with that or GA or auxin. In other words, they contained many more down-regulated genes than up-regulated genes considering both 48 hpi WT versus MU and WT 48 hpi versus 6 hpi, indicating their positive roles in plant disease defense, which is in agreement with previous reports (Broekaert et al., 2006).

Others. Other plant hormones, such as salicylic acid (SA) and brassinosteroids (BAs) and their signaling were also broadly reported to be involved in the modulation of plant defense response. However, very few DE genes were fall into these two classes. One explanation for the SA category not being identified in this study is that these genes are generally involved in systemic resistance.

Photosynthesis

Notably, the photosynthesis category was tremendously down-regulated following infiltration with Xcc306 relative to that with mutant Xcc306ΔpthA4 at both 48 hpi and 120 hpi as well as in the scenario with WT infection at 48 hpi versus at 6 hpi (Table 3-1, 2-3, Figure 3-5, 2-8A, 2-9C). The expression of global photosynthesis related genes were well documented to be repressed upon pathogen assault in

previous studies (Zou et al., 2005; Bonfig et al., 2006; Bilgin et al., 2010; Fu et al., 2012). The regulatory sets of the photosynthesis genes also include genes coding for electron transport, ATP synthase and carbon metabolism, some of which were also shown to be down-regulated (Table 3-3, 2-5, Figure 3-7A). The decreased rate of photosynthesis was associated with an early increase in cell wall invertase activity, which cleaved sucrose in the apoplast into hexoses (glucose and fructose), leading to an accumulation of hexoses (Swarbrick et al., 2006; Berger et al., 2007); this was in agreement with the induction of sucrose metabolism related genes. Most of the down-regulated photosynthesis gene products target to the chloroplast and thylakoid. The reduction of photosynthesis proteins is regarded as the adaptive consequences of pathogen attack rather than a contributor to plant vulnerability, since it also uniformly happened following other biotic or abiotic stresses (such as drought, salinity and temperature). Photosynthate is the energy source for both the plant and pathogen. After attack, the plant requires the investment of energy supply from the photosynthetic pathway to be redirected to defense machinery supporting plant fitness.

Plant Defense Components

Plant defense components were defined as the plant defense response indictors, the secondary metabolism products that prevent pathogen invasion.

PR proteins. Accumulating evidences has shown that the plant PR (pathogenesis related) proteins were antimicrobial compounds and played critical roles in plant defense rather than in pathogenesis (Ryals et al., 1996; Sels et al., 2008; Fu and Dong, 2013); they are generally considered as markers for defense response to biotic stimulus. The expressions of *PR-1* (unknown functions), *PR-2* (encoding β-1, 3-glucanase) and *PR-5* (encoding a thaumatin-like protein), were induced during the

onset of salicylic acid-mediated systemic acquired resistance (SAR), acting as readouts of SAR. Proteinase inhibitors (PIs), member of the PR6 family, play essential roles in plant defense response against pathogens by compromising their ability (Solomon et al., 1999; Sels et al., 2008). The expression of most putative *PR* gene families in *C. sinensis* leaves were suppressed following infection of *Citrus leprosis virus* (CiLV), while few were induced, although the patterns varied in the response of different pathogens as well as different citrus species (Campos et al., 2007; Khalaf et al., 2011). From the transcriptional profiles in the comparison of WT and MU infection at 48 hpi and the comparison of 48 hpi vs. 6 hpi by WT infection, the PR protein category, which contained abundant repressed genes, was significantly down-regulated (Table 3-1, 2-4).

Phenylpropanoids and PAL. Another group of defense-related compounds is phenylpropanoids, which function as antimicrobial compounds. These are well known for playing important roles in resistance to pathogen attack (Dixon et al., 2002). Phenylpropanoid derivatives include lignin, lignans, coumarins and flavonoids. Phenylalanine-ammonia lyase (PAL) is the key branch point enzyme for the biosynthesis of phenylpropanoids from phenylalanine, and is widely known to be induced upon pathogen attack (Dixon and Paiva, 1995). The biosynthesis of flavonoids complex requires multiple enzymes including chalcone synthase (CHS), cytochrome P450 monooxygenases etc., and flavonoid pathway genes in several species were activated in defence responses elicited by different pathogens (Naoumkina et al., 2010). In our study, a considerable number of genes that are involved in phenylpropanoids metabolism (especially lignin biosynthesis) were down-regulated in all three comparisons of WT 48 hpi vs. 6 hpi, 48 hpi WT vs. MU and 120 hpi WT vs. MU (20, 21

and 28 genes involved respectively) (Table 3-2). In addition, the category of phenylpropanoid biosynthetic processes was significantly down-regulated at both 48 hpi and 120 hpi when compared to the wild-type and mutant inoculation. Also, the KEGG pathway analysis also showed the remarkably down-regulation of flavonoid biosynthesis and phenylpropanoid biosynthesis pathways at 48 hpi.

Categories Associated with Basal Defense

Protein ubiquitination (especially by E3 ubiquitin ligase) and degradation has been implicated in plant defense response either accomplished by PTI (PAMPs-triggered immunity) or ETI (effector-triggered immunity) (Yang et al., 2006b; Trujillo et al., 2008). The plant employ an intricate ubiquitin-proteasome system to recognize and degrade the foreign effector molecules (Craig et al., 2009). To interfere with plant immunity, bacterial effectors need to destroy some key factors in this system. The silencing of genes encoding cellular ubiquitin, which involved the proteasomal protein degradation pathway, induce extreme susceptibility of the transformed barley (*Hordeum vulgare*) towards the host fungus pathogen *Blumeria graminis* f. sp *hordei* that cause powdery mildew (Dong et al., 2006). In the presence of PthA4, many genes that participate in ubiquitin degradation were up-regulated.

Receptor-like kinases (RLKs), which are critical for the recognition of pathogen-associated molecular patterns (PAMPs) like bacterial flagellin and elongation factor (EF-Tu), are involved in basal defense and triggering plant immunity response. To date, typically almost all confirmed RLKs that contain a non-arginine-aspartate (non-RD) kinase motif are involved in the recognition of multiple conserved microbial signatures and confer broad-spectrum resistance (Schwessinger and Ronald, 2012). The receptor kinase class is extremely over-represented in the type III effector-suppressed data sets

(Truman et al., 2006). A receptor-like kinase protein, XA21 in rice (*Oryza sativa*), was revealed to confer gene-for-gene resistance to *X. oryzae* pv *oryzae*, the causal agent of bacterial blight, through interacting with an E3 ubiquitin ligase XB3 (XA21 binding protein 3) (Wang et al., 2006). From Figure 3-8B and Figure 3-12, although a substantial number of DE genes was assigned to receptor kinases category, the number of up-regulated genes and down-regulated genes included did not greatly differ.

Binding

Binding is an important category in respect to molecular function. By comparing of 48 hpi WT versus MU, the binding group contained a large number of DE genes, while the percentage of up-regulated genes involved in binding was much higher than that of down-regulated genes (Figure 3-3, 2-6). In more detail, the up-regulated genes were mainly enriched in copper ion binding, while the down-regulated genes were primarily enriched in heme-binding (Table 3-2).

Copper ion binding. Copper is an important agent in growth of *Xanthomonas* spp. It diminishes their population on leaf surfaces, while copper-based bactericides have been an effective control measure for citrus canker (Graham et al., 2004). *X. oryzae* pv. *oryzae* (Xoo) strain PXO99 uses the susceptibility protein Xa13 cooperatively with two other proteins, COPT1 and COPT5 to remove copper from leaf surface and promote its multiplication and spread; the modulation of copper redistribution was proposed as a mechanism for bacterial virulence (Yuan et al., 2010). Copper ion binding activity may be a part of copper redistribution to protect the plant from bacterial invasion or sponsor the colonization of bacteria. In three comparisons, WT 48 hpi versus 6 hpi, 48 hpi WT versus MU and 120 hpi WT versus MU, the class of copper ion binding was significantly up-regulated in SEA test (Table 3-2, Table 3-5).

ATP binding. ATP binding cassette (ABC) transporters are pathogen induced (few repressed) and required for resistance responses to pathogens. They direct the transport of plant metabolites out of the local cell in order to restrict the pathogen in the apoplast (Wanke and Kolukisaoglu, 2010). Arabidopsis ABC transporter AtPEN3/PEN8, contributes to non-host resistance to *B. graminis* and *Phytophthora infestans* by halting fungal penetration (Stein et al., 2006). ATP binding category was found to be significantly up-regulated at 48 hpi by WT relative to 6 hpi.

Other bindings. Heme-binding may be involved in cytochrome P450 activities(Li et al., 2008). Limited knowledge about the relevance of zinc and other cation ion binding to pathogen attack is available and remains to be determined.

Other Important Categories

Lipid metabolism. Lipids are basic components of plant cell membranes and provide fundamental energy for metabolic activities. Lipid metabolism was regarded as an important pathway in plants responding to pathogens (Shah, 2005). JA, one of the best-studied signal molecules shown to be critical in the plant response to disease, is a lipid-derived metabolite. One of the lipid component, fatty acids (FAs), work as source of reserve energy and, are mainly considered to play passive roles in plant pathogen defense while their breakdown products are able to induce defense response (Kachroo and Kachroo, 2009). Overexpression of Arabidopsis gene *ACBP3*, which serves critical roles in lipid metabolism, enhanced plant resistance to *Pseudomonas syringe* pv. *tomato* DC3000 (Xiao and Chye, 2011). In this study, the lipid biosynthetic process and in particular FA synthesis was significantly down-regulated in 48 hpi and 120 hpi WT versus MU. This is an indication that these type of genes may be beneficial to plant defense (Table 3-2, Figure 3-7).

UDP-glycosyl transferases. UDP-glycosyl transferases (UGTs) belong to the family of glycosyltransferases that catalyze the transfer of nucleotide-diphosphate-activated sugars to secondary metabolites, leading to glycosylation (Vogt and Jones, 2000). UGTs play important and positive roles in plant-pathogen interactions. Two glycosyltransferase genes *UGT73B3* and *UGT73B5* in Arabidopsis, were notably induced following infection of *Pseudomonas syringae* pv tomato, while the mutation of these two genes exhibited decreased resistance to this pathogen (Langlois-Meurinne et al., 2005). This category was also down-regulated by WT relative to MU at 48 hpi (Table 3-2).

Cell cycle and division. In strong accordance with the hypertrophy and hyperplasia of the cells caused by Xcc306, the expression of cell cycle, development and cell division related genes were altered preferentially in response to wild-type Xcc306 than to *pthA4* deletion mutant. The ribosomal protein biosynthesis process was regarded to correlate with cell division. Nucleosome assembly protein (NAP1) is involved in cell cycle control, which promotes cell proliferation and cell expansion during Arabidopsis leaf development (Galichet and Gruissem, 2006). The cytoskeleton proteins, microtubule-associated proteins, were considered to be involved in progression of cytokinesis. These categories were up-regulated in 48 hpi and 120 hpi in respect to WT versus MU (Table 3-1, 2-2, Figure 3-4).

Molecular Events at Early Time of Infection and From Earlier Time to Later Time

At 6 hpi, very few genes were up-regulated when comparison were made between WT and MU infection; otherwise, lots of genes were down-regulated. Astonishingly, from SEA categorization, the down-regulated genes were significantly assigned to categories potentially associated with plant defense, given that apart from

the well-studied gene classes (like innate immune response, programmed cell death, ubiquitin ligase), most of the other categories were also observed to be repressed at 48 hpi and 120 hpi (Table 3-2). This demonstrated that PthA4 protein molecules were delivered as early as 6 hours after infiltration to assault plant basal defense system, but the more complicated pathogenic systems to incite ETS were not established yet at this time point.

Table 3-1. Categories with expression significantly changed (*P*-value<0.05) and corresponding DE probesets number by the infection of wild-type Xcc306 relative to by mutant Xcc306Δ*pthA4* at 48 hpi generated by MapMan software

Functional categories	Elements number	*P*-value
48 hpi WT vs. MU up-regulated	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}
Beta 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 physiology	2	4.0×10^{-2}
48 hpi WT vs. MU down-regulated	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-PR proteins	25	2.6×10^{-11}
Protein postranslational 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}

Table 3-2. Significantly altered GO terms (FDR<0.05) according to gene expression and contained DE genes number upon the comparison between wild-type and mutant infection at 6 hpi, 48 hpi and 120 hpi through Singular Enrichment Analysis

Enrichment terms	6 hpi WT vs. MU down (190)	48 hpi WT vs. MU up (590)	48 hpi WT vs. MU down (479)	120 hpi WT vs. MU up (724)	120 hpi WT vs. MU down (893)
Biological process	Innate immune response (9)	Protein-DNA complex assembly (10)	Secondary metabolic process (35)	Protein-DNA complex assembly (8)	Secondary metabolic process (45)
	Programmed cell death (6)	DNA packaging (11)	Aromatic compound biosynthetic process (31)	DNA packaging (8)	Aromatic compound biosynthetic process (23)
	Defence response to bacterium (6)	DNA replication (17)	Monocarboxylic acid metabolic process (26)	DNA replication (15)	Monocarboxylic acid metabolic process (38)
		Protein polymerization (5)	Cellular amino acid derivative biosynthetic (27)	Regulation of cell cycle (8)	Reproductive process in a multicellular organism (5)
		Microtubule-based movement (11)	Phenylpropanoid biosynthetic process (18)	Microtubule-based movement (19)	Phenylpropanoid biosynthetic process (23)
	Carboxylic acid biosynthetic process (12)	Plant-type cell wall organization (12)	Carboxylic acid biosynthetic process (26)	Plant-type cell wall organization (8)	Protein-chromophore linkage (5)
		Cell wall modification (5)	Lipid biosynthetic process (19)	Lipid localization (5)	Lipid metabolic process (44)
	Heterocycle biosynthetic process (7)	Carbohydrate metabolic process (43)	Heterocycle biosynthetic process (14)	Heterocycle catabolic process (6)	Photosynthesis (33)
		Sucrose metabolic process (14)	L-phenylalanine metabolic process (11)	Sucrose metabolic process (25)	

Table 3-2. Continued.

Enrichment terms	6 hpi WT vs. MU down (190)	48 hpi WT vs. MU up (590)	48 hpi WT vs. MU down (479)	120 hpi WT vs. MU up (724)	120 hpi WT vs. MU down (893)
Biological process		Starch metabolic process (14)	Electron transport (26)	Starch metabolic process (25)	
			Carbohydrate transport (7)	Cell division (14)	
				Nuclear division (7)	
				Response to gibberellin stimulus (9)	
Molecular function	Carboxylesterase activity (8)	Copper ion binding (8)	Peptidase inhibitor activity (9)	Copper ion binding (11)	Carboxylesterase activity (22)
	Oxidoreductase activity (30)	Enzyme regulator a Ctivity (14)	Oxidoreductase activity (53)	Enzyme inhibitor activity (13)	Oxidoreductase activity (91)
	Carbohydrate binding (6)	Pectate lyase activity (5)	Monooxygenase activity (17)	Hydrolase activity on glycosyl compounds (28)	Monooxygenase activity (26)
	Ubiquitin-protein ligase activity (5)	Pectinesterase activity (7)	UDP-glycosyltransferase activity (14)	Pectinesterase activity (9)	Transferase activity, transferring glycosyl groups (30)
	FAD binding (6)	DNA-dependent atpase activity (6)	Iron ion binding-heme binding (11)	DNA-dependent atpase activity (7)	Heme binding (20)
	Lyase activity (9)	Motor activity (6)	Lyase activity (22)	Microtubule motor activity (5)	Lyase activity (29)
			Sugar transmembrane transporter activity (6)		Transferase activity, transferring acyl groups and amino-acyl groups (18)

Table 3-2. Continued.

Enrichment terms	6 hpi WT vs. MU down (190)	48 hpi WT vs. MU up (590)	48 hpi WT vs. MU down (479)	120 hpi WT vs. MU up (724)	120 hpi WT vs. MU down (893)
Molecular function					Oxidosqualene cyclase activity (5)
Cellular component	Ubiquitin ligase complex (5)	Endomembrane system (38)	Thylakoid (24)	Endomembrane system (50)	Thylakoid (71)
	Cell wall (7)	Anchored to membrane (8)	Organelle subcompartment (16)	Anchored to membrane (11)	Organelle subcompartment (34)
		Cell wall (15)	Cell wall (18)	Cell wall (19)	Integral to membrane (54)
		Extracellular region (24)	Photosystem (6)	Extracellular region (23)	Photosystem (20)
		Cytoplasmic vesicle (56)		Cytoplasmic vesicle (69)	Chloroplast (95)
		Cytoskeleton (19)		Cytoskeleton (22)	
		Protein-DNA complex (8)		Protein-DNA complex (6)	
		Chromosome (11)			

Note: up and down represent up-regulated and down-regulated upon the corresponding comparisons.

Table 3-3. Significantly changed categories in expression (*P*-value<0.05) and contained DE probesets number by the infection of wild-type Xcc306 relative to by mutant Xcc306Δ*pthA4* at 6 hpi and 120 hpi generated by MapMan software

Function categories	Elements number	*P*-value
6 h WT vs. MU up-regulated	29 (no significant category)	
6 h WT vs. MU down-regulated	233	
Auxin responsive	2	1.5×10^{-2}
Acid and other phosphatases	3	9.5×10^{-3}
120 h WT vs. MU up-regulated	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}
120 h WT vs. MU down-regulated	1094	
Photosynthesis	73	5.8×10^{-2}
Lightreaction	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 (LTP)	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}
Postranslational 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}

Table 3-3. Continued.

Function categories	Elements number	*P*-value
Cell	17	3.0×10^{-3}
Cell organisation	9	8.8×10^{-3}
Glycolysis	4	1.1×10^{-2}
TCA transformation	3	3.3×10^{-2}

Table 3-4. Categories with significantly changed in expression (*P*-value<0.05) and contained DE probesets number through the comparisons of different time after infiltration of wild-type Xcc306 or mutant Xcc306Δ*pthA4* generated by MapMan software

Function categories	Elements number	*P*-value
MU 48 hpi vs. 6 hpi up-regulated	964	
Lipid metabolism	9	3.0×10^{-2}
Lipid degradation	4	8.4×10^{-3}
Lignin biosynthesis	4	2.6×10^{-2}
Auxin-responsive	4	5.6×10^{-3}
Stress	75	3.2×10^{-5}
Biotic stress	23	1.1×10^{-3}
Abiotic stress	51	7.8×10^{-5}
Heat stress	41	5.2×10^{-6}
Nucleotide metabolism	2	4.1×10^{-3}
Miscellaneous enzymes	95	1.5×10^{-2}
Cytochrome P450	22	2.3×10^{-2}
Nitrile lyases	7	3.1×10^{-2}
MYB-related transcription factor	7	3.2×10^{-3}
C2C2 (Zn)-like zinc finger family	5	1.2×10^{-3}
DNA synthesis	3	4.0×10^{-2}
Protein	64	3.6×10^{-4}
Postranslational modification	15	1.7×10^{-4}
E3 ubiquitin	15	3.0×10^{-3}
Cell division	5	2.3×10^{-3}
Development	28	4.0×10^{-2}
MU 48 hpi vs. 6 hpi down-regulated	902	
Cell wall precursor synthesis	4	3.0×10^{-2}
Cell wall protein	5	2.8×10^{-2}
Lipid transfer proteins	4	4.2×10^{-2}
Secondary metabolism-chalcones	6	1.2×10^{-2}
Auxin responsive	7	3.0×10^{-2}
Vitamine metabolism-thiamine	4	5.5×10^{-3}
Starch degradation	2	3.1×10^{-2}
PR-proteins	4	5.0×10^{-2}
Drought/salt stress	7	7.2×10^{-3}
Redox regulation	7	3.5×10^{-3}
DNA	5	4.4×10^{-2}

Table 3-4. Continued.

Function categories	Elements number	*P*-value
Protein	38	3.5×10^{-2}
G-protein signal	3	4.7×10^{-2}
WT 48 hpi vs. 6 hpi up-regulated	1147	
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 figure 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}
Sulphate transport	3	2.5×10^{-3}
WT 48 hpi vs. WT 6 hpi down-regulated	1119	
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}
PR-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}
MU 120 h vs. MU 48 h up-regulated	136	
Oxidases	3	1.2×10^{-2}
Receptor kinases	2	4.7×10^{-2}
MU 120 h vs. MU 48 h down-regulated	237	
Lipid degradation	4	2.9×10^{-3}
Jasmonate metabolism	2	2.5×10^{-2}
Stress	40	2.2×10^{-2}
PR-proteins	17	2.2×10^{-3}
Proteinase inhibitors	14	1.1×10^{-4}
Cell organisation	3	2.7×10^{-2}

Table 3-4. Continued.

Function categories	Elements number	*P*-value
WT 120 h vs. WT 48 h up-regulated	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}
WT 120 h vs. WT 48 h down-regulated	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}
Phospholipid synthesis	3	3.0×10^{-2}
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}

Table 3-5. Significantly DE genes enriched GO terms (FDR<0.05) according to the comparison of different hours post infiltration of wild-type or mutant through Singular Enrichment Analysis

Enrichment terms	MU 48 hpi vs. 6 up	MU 48 hpi vs. 6 hpi down	WT 48 hpi vs. 6 h up	WT 48 hpi vs. 6 hpi down	MU 120 hpi vs. 48 hpi up	MU 120 hpi vs. 48 hpi down	WT 120 hpi vs. 48 hpi up	WT 120 hpi vs. 48 hpi down
Biological process	Secondary metabolic process		Chitin catabolic process	Aromatic compound biosynthetic process			Chitin catabolic process	
	Chitin and glucose catabolic process	Starch metabolic process	Starch metabolic process				Starch metabolic process	Glucan metabolic process
	Carbohydrate metabolic process	Sucrose metabolic process	Sucrose metabolic process				Starch metabolic process	
	Cellular nitrogen compound metabolic process	Flavonoid biosynthetic process	Cellular nitrogen compound metabolic process	Flavonoid biosynthetic process			Cellular nitrogen compound metabolic process	
	Phenylpropanoid biosynthetic process	Lipid metabolic process	DNA packaging	Lipid metabolic process				Monooxygenase activity
	Pigment metabolic process	Heterocycle biosynthetic process	Protein-DNA complex assembly	Electron transport				Lipid transport

Table 3-5. Continued.

enrichment terms	MU 48 hpi vs. 6 up	MU 48 hpi vs. 6 hpi down	WT 48 hpi vs. 6 h up	WT 48 hpi vs. 6 hpi down	MU 120 hpi vs. 48 hpi up	MU 120 hpi vs. 48 hpi down	WT 120 hpi vs. 48 hpi up	WT 120 hpi vs. 48 hpi down
Biological process	Mannose, fructose, glyoxylate and inositol metabolic process	Defence response, incompatible interaction	DNA-dependent DNA replication				Microtubule-based movement	Peptide transport
	Response to heat		Response to heat			Response to heat	Mitosis	Response to heat
	Response to inorganic substance		Response to inorganic substance	Response to chemical stimulus			Response to oxidative stress	Response to auxin stimulus
	Response to light intensity		Plant-type cell wall organization				Cell cycle	Developmental growth
			Cell division				Cell division	
			Cell wall macromolecule catabolic process				Cell wall macromolecule catabolic process	
Molecular function	Glucosyltransferase activity	Pectate lyase activity	Plant-type cell wall organization	Transcription factor activity		UDP-glycosyltransferase activity		

Table 3-5. Continued.

enrichment terms	MU 48 hpi vs. 6 up	MU 48 hpi vs. 6 hpi down	WT 48 hpi vs. 6 h up	WT 48 hpi vs. 6 hpi down	MU 120 hpi vs. 48 hpi up	MU 120 hpi vs. 48 hpi down	WT 120 hpi vs. 48 hpi up	WT 120 hpi vs. 48 hpi down
Molecular function	Oxidoreductase activity	Monooxygenase activity	DNA-dependent atpase activity	Monooxygenase activity	Oxidoreductase activity	Dioxygenase activity	Peroxidase activity	Monooxygenase activity
	Chitinase activity		Chitinase activity	Oxidoreductase activity		Hydro-lyase activity	Chitinase activity	
	Carbohydrate phosphatase activity	Carboxylesterase activity	ATP binding	Endopeptidase inhibitor activity		Endopeptidase inhibitor activity	Endopeptidase inhibitor activity	Endopeptidase inhibitor activity
	Chitin binding	Heme binding	Chitin binding	Heme binding	Heme binding		Heme binding	Carboxylesterase activity
	Unfolded protein binding	Carbohydrate binding	Copper ion binding		Ion binding			
	Transmembrane transporter activity		Water transmembrane transporter activity	Structural constituent of cell wall				Structural constituent of cell wall
Cellular component	N/A	Cell wall Anchored to membrane	Cell wall Anchored to membrane	Cell wall	N/A	Cell wall		Cell wall Endomembrane system

Table 3-5. Continued.

enrichment terms	MU 48 hpi vs. 6 up	MU 48 hpi vs. 6 hpi down	WT 48 hpi vs. 6 h up	WT 48 hpi vs. 6 hpi down	MU 120 hpi vs. 48 hpi up	MU 120 hpi vs. 48 hpi down	WT 120 hpi vs. 48 hpi up	WT 120 hpi vs. 48 hpi down
Cellular component		Cytoplasmic membrane-bounded vesicle	Cytoplasmic membrane-bounded vesicle Extracellular region Cytoskeleton Protein-DNA complex				Cytoplasmic membrane-bounded vesicle Extracellular region Microtubule cytoskeleton	Cytoplasmic membrane-bounded vesicle Extracellular region

Note: up and down represent up-regulated and down-regulated upon the corresponding comparisons.

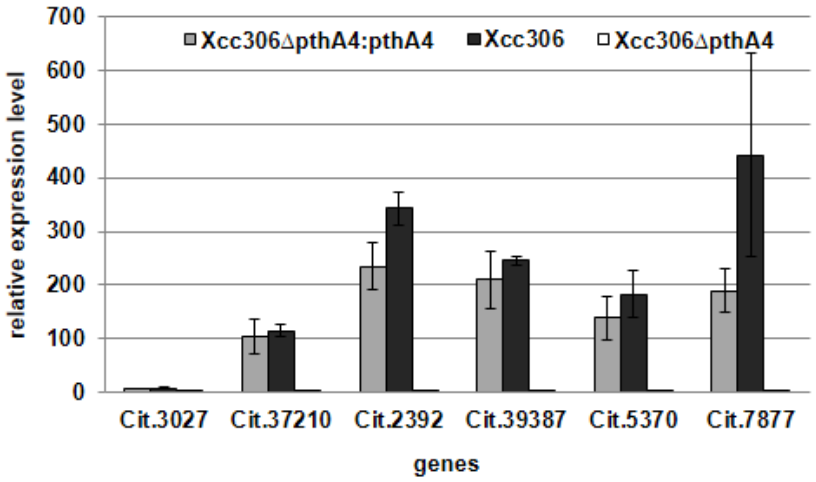


Figure 3-1. Quantitative RT-PCR validation of selected up-regulated genes in Microarray analysis. The sweet orange leave tissues were sampled 48 h after the infiltration of wild-type Xcc306, Xcc306Δ*pthA4* as well as the complement strain Xcc306Δ*pthA4*:PthA4. Data represent the mean ± SD. The labels represent Affymetrix probe IDs Cit.3027.1.S1_s_at, Cit.37210.1.S1_at, Cit.2392.1.S1_at, Cit.39387.1.S1_at, Cit.5370.1.S1_s_at and Cit.7877.1.S1_at respectively.

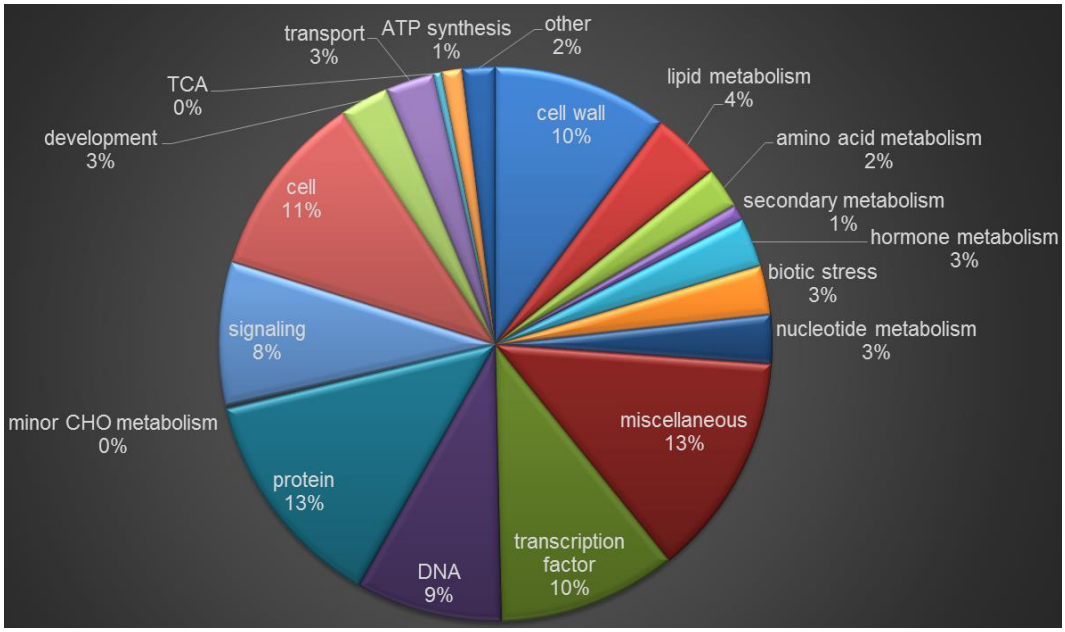


Figure 3-2. Pie chart demonstrating the proportions of the functional categories formed from MapMan that contain up-regulated genes by infection of Xcc306 relative to mutant Xcc306Δ*pthA4* at 48 hpi. The 'miscellaneous' class includes various enzymes such as cytochrome P450, invertase, protease inhibitor, glucan hydrolases, nitrilase, oxidases and so on. The 'other' category includes major CHO metabolism, redox, glycolysis and fermentation. The genes with unassigned were eliminated.

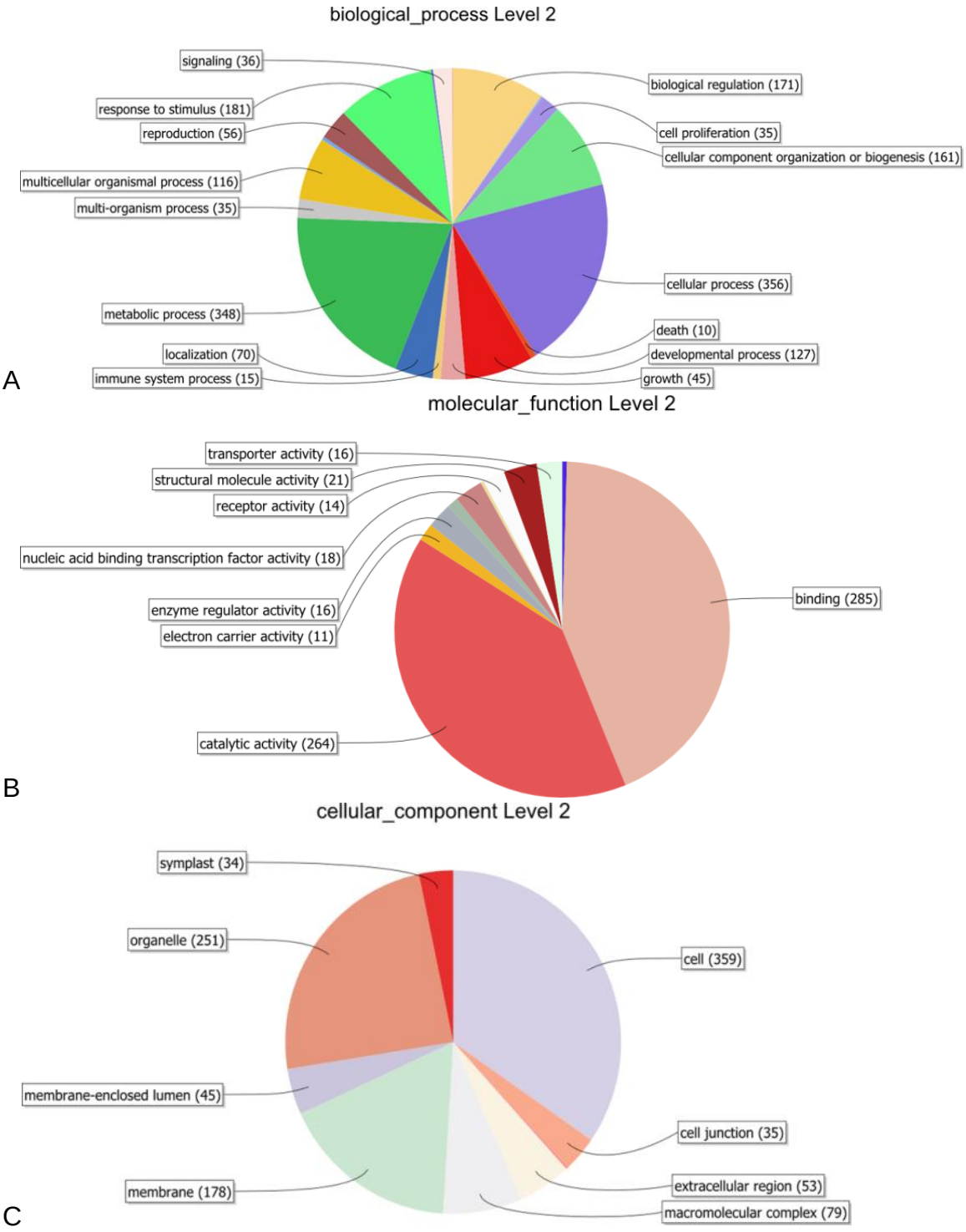


Figure 3-3. Pie chart showing the proportions of the second level GO terms formed from BLAST2GO that contain the up-regulated genes by infection of Xcc306 relative to mutant Xcc306Δ*pthA4* at 48 hpi. The GO terms were divided from three large groups, biological process (A), molecular function (B) and cellular component (C). The number of up-regulated genes in each category is also displayed.

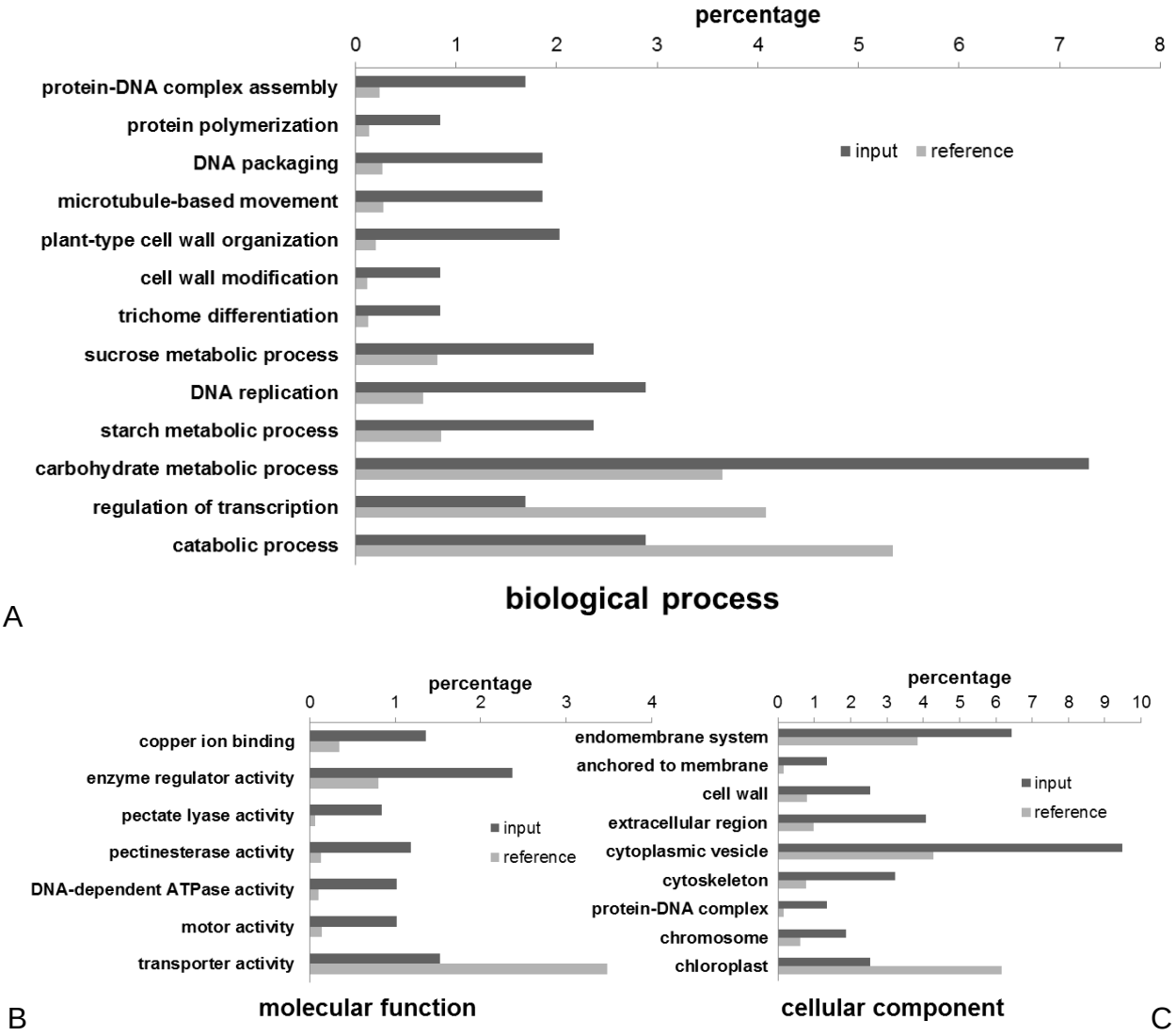


Figure 3-4. Some of over-represented and under-represented categories in up-regulated gene sets by infection of Xcc306 relative to mutant Xcc306Δ*pthA4* at 48 hpi in Singular Enrichment Analysis (FDR<0.05). The 'input' legend (light black bar) represents the percentage out of total up-regulated genes, 'reference' (dark white bar) represents the percentage of genes in individual category out of total citrus ESTs number. The categories with 'input' significantly exceeding 'reference' are thought to be over-represented, and vice-versa. The genes were categorized based on biological process (A), molecular function (B) and cellular component (C).

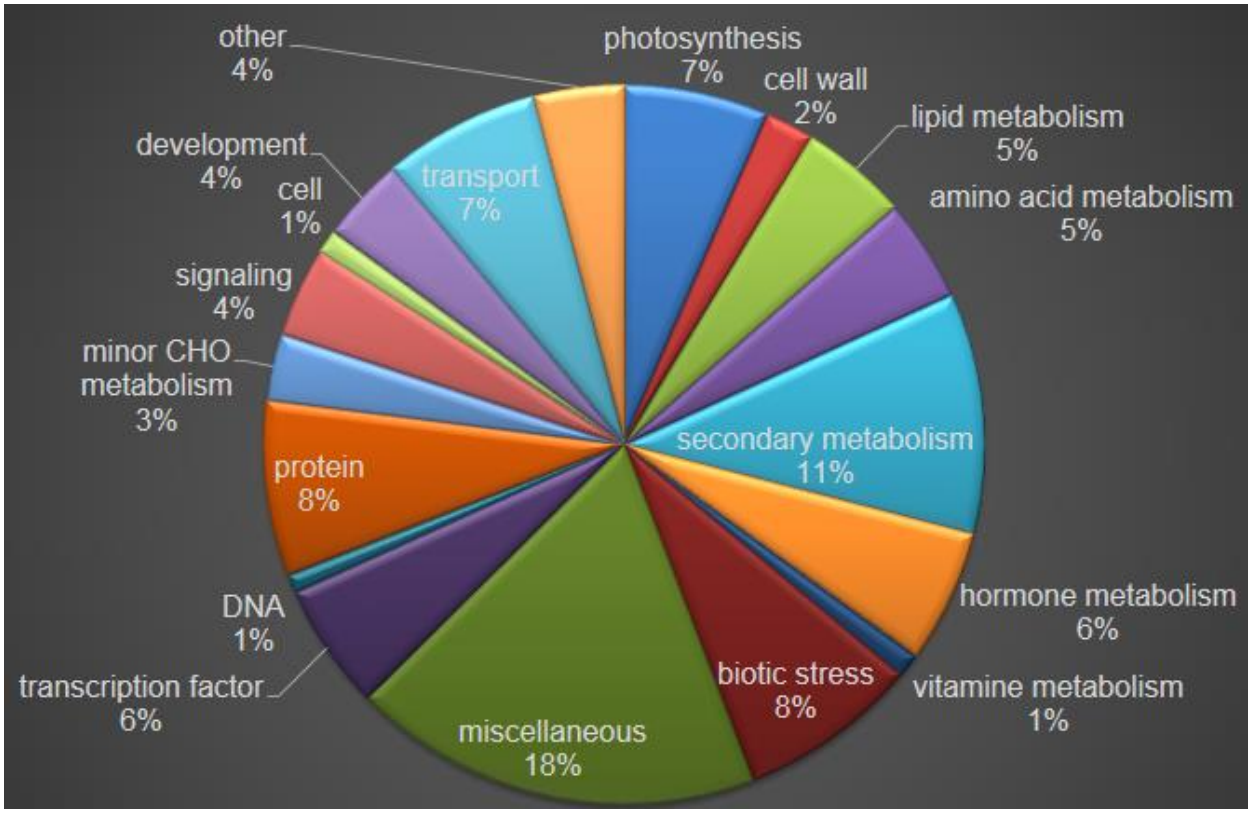


Figure 3-5. Pie chart demonstrating the proportions of the functional categories formed from MapMan that contain down-regulated genes by infection of Xcc306 relative to mutant Xcc306Δ*pthA4* at 48 hpi. The 'miscellaneous' class mainly includes various enzymes such as cytochrome P450, UDP glucosyl and glucoronyl transferases, nitrilase, oxidases and so on. The 'other' category includes major CHO metabolism, redox, glycolysis and fermentation. The genes with unassigned were excluded.

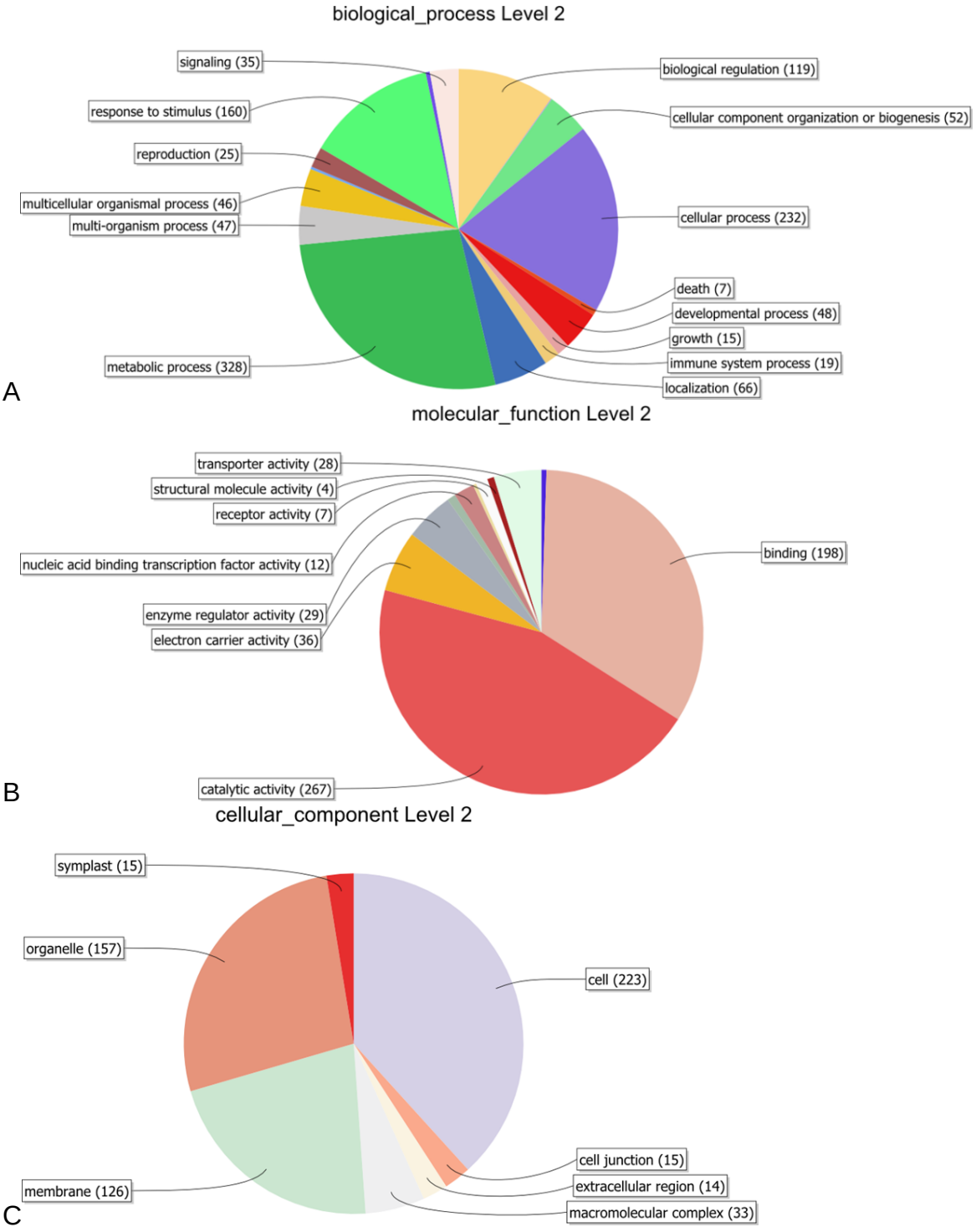


Figure 3-6. Pie chart showing the proportions of the second level GO terms formed from BLAST2GO that contain the down-regulated genes by infection of Xcc306 relative to mutant Xcc306Δ*pthA4* at 48 hpi. The GO terms were divided from three large groups, biological process (A), molecular function (B) and cellular component (C). The number of down-regulated genes in each category is also displayed.

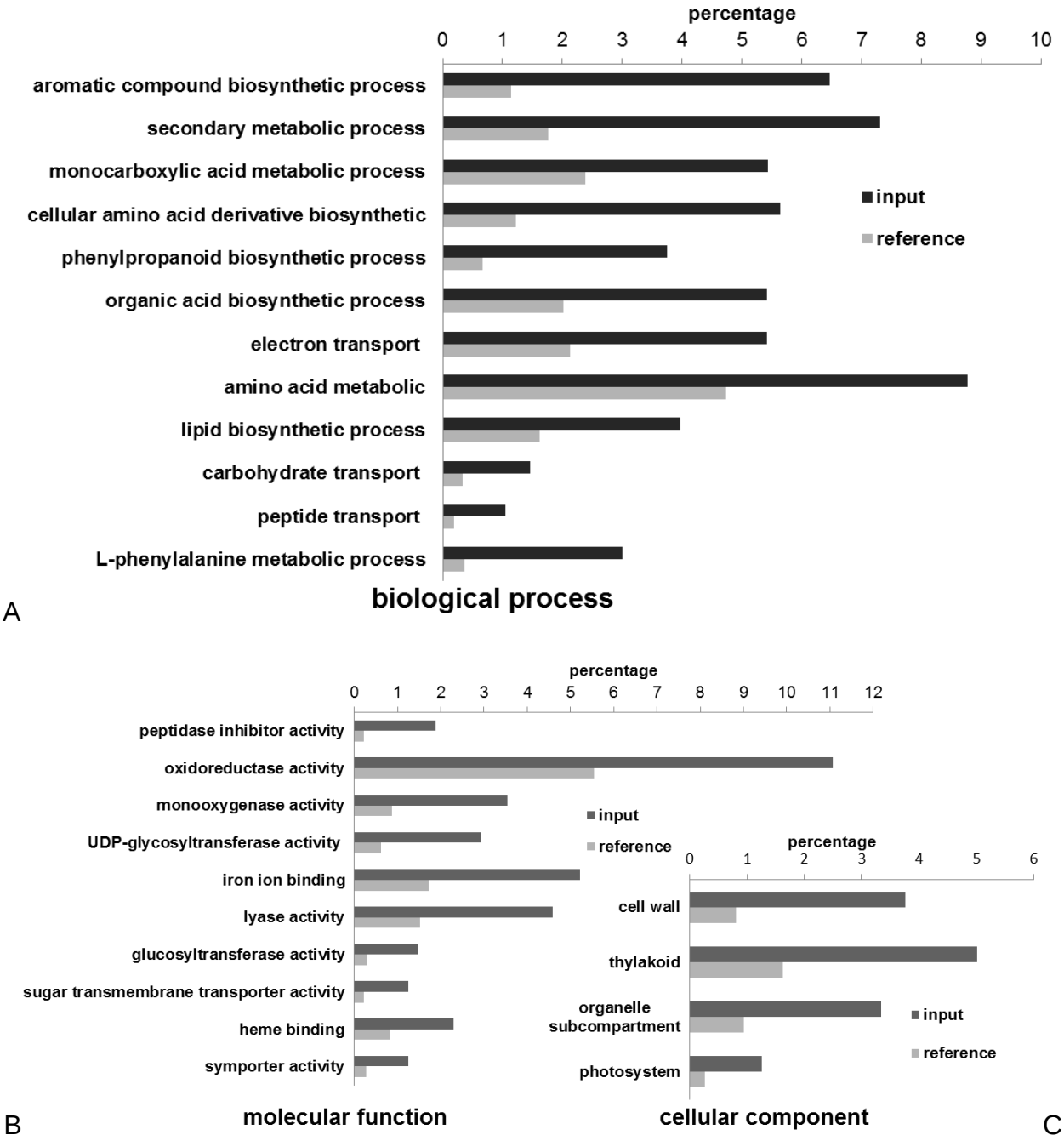
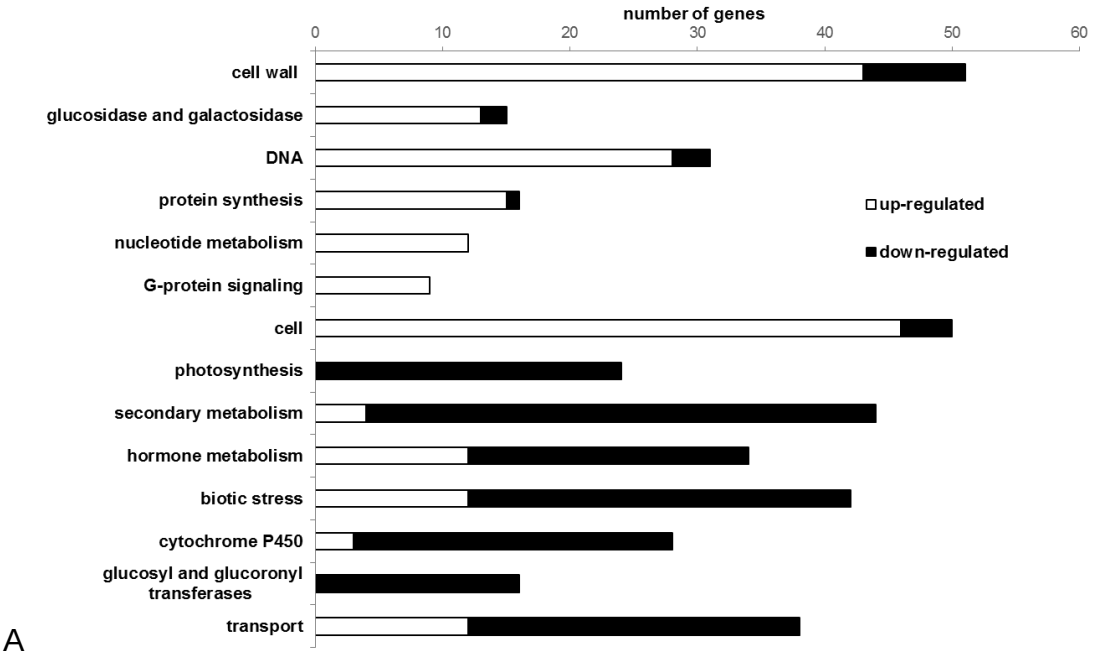
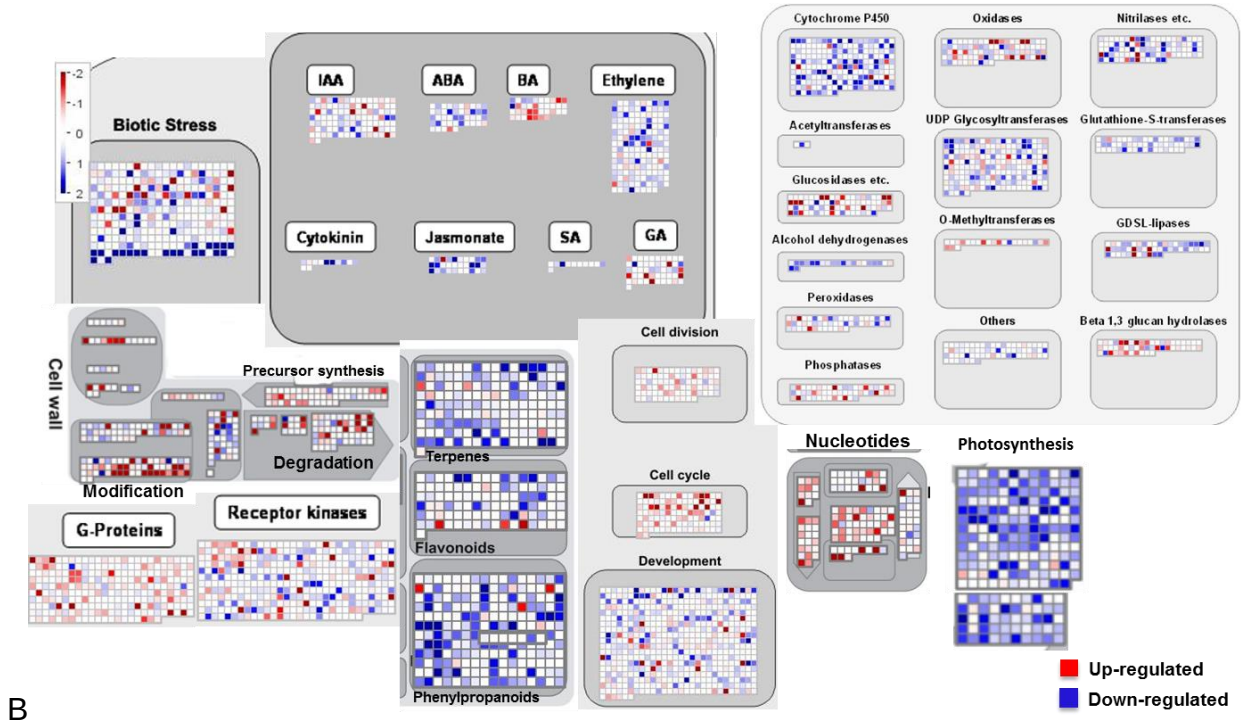


Figure 3-7. Categories that significantly contain more percentage of down-regulated genes considering 48 hpi WT versus MU than their proportions out of total categories calculated by Singular Enrichment Analysis (FDR<0.05). The 'input' legend represents the percentage in total down-regulated genes, 'reference' represents the percentage of genes in individual category out of total citrus ESTs number. The genes were categorized based on biological process (A), molecular function (B) and cellular component (C).

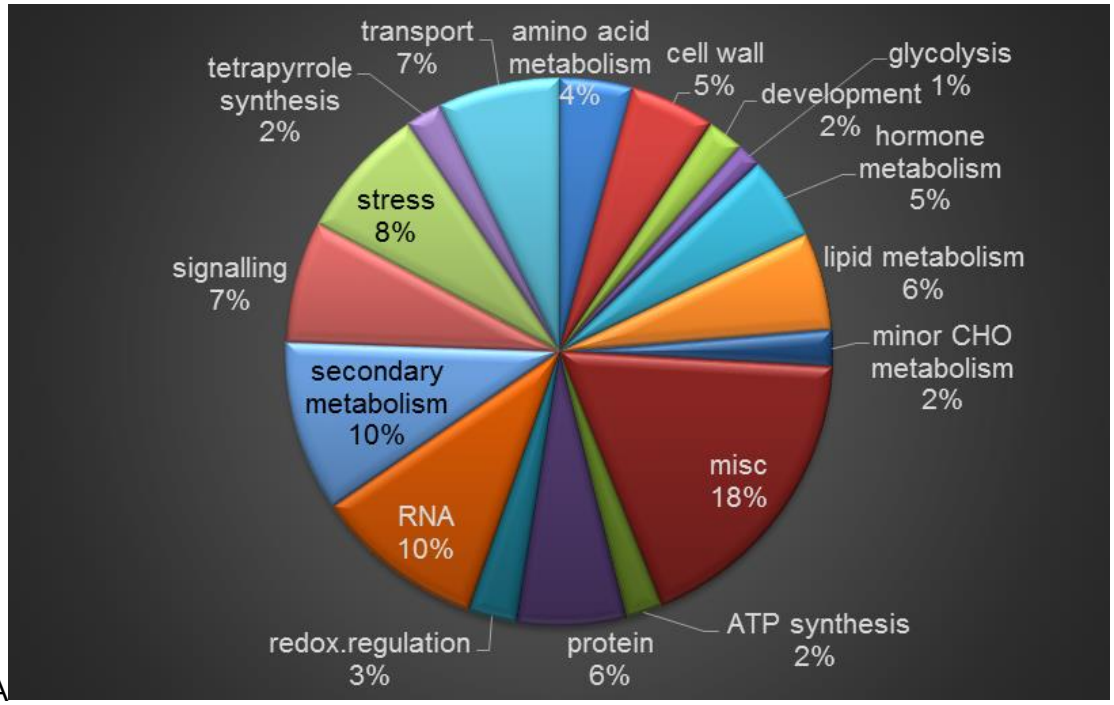


A

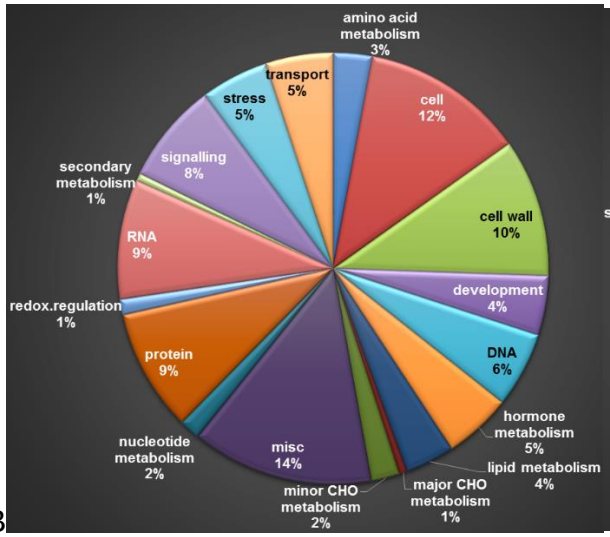


B

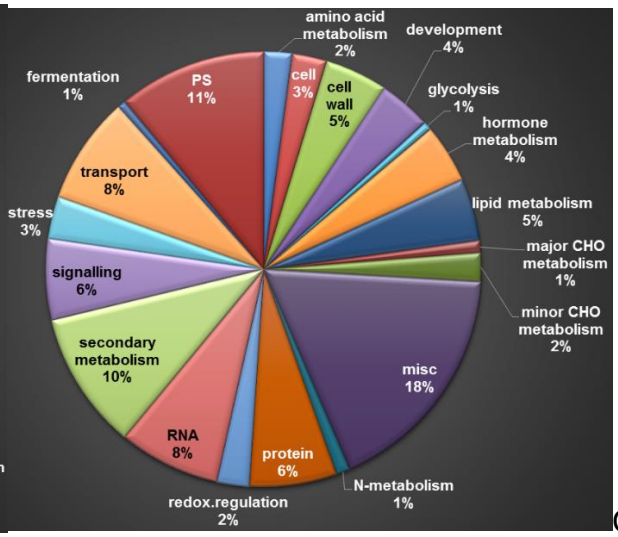
Figure 3-8. Comparison of up-regulated and down-regulated genes number regarding 48 hpi WT versus MU in some categories basing on the categorization by MapMan. A) Bar chart showing the categories with extraordinary different amount of up-regulated genes (white bars) and down-regulated (black bars). B) The up-regulated genes and down-regulated genes assigned to some important categories are visualized by MapMan software. The scale with log2 fold change is shown.



A



B



C

Figure 3-9. Pie chart demonstrating the proportions of the functional categories formed from MapMan that contain DE genes by infection of Xcc306 relative to mutant Xcc306Δ*pthA4* at 6 hpi and 120 h. A) Category partition of down-regulated gene data set at 6 hpi. B) Percentages of each individual category regarding up-regulated genes number contained at 120 hpi. C) Percentages of each individual category in respect to down-regulated genes number contained at 120 hpi. The 'misc' class mainly includes various enzymes such as cytochrome P450, peroxidases, UDP glucosyl and glucoronyl transferases, gluco-, galacto- and mannosidase, protease inhibitor, nitrilase, oxidases and so on. The genes with unassigned were excluded.

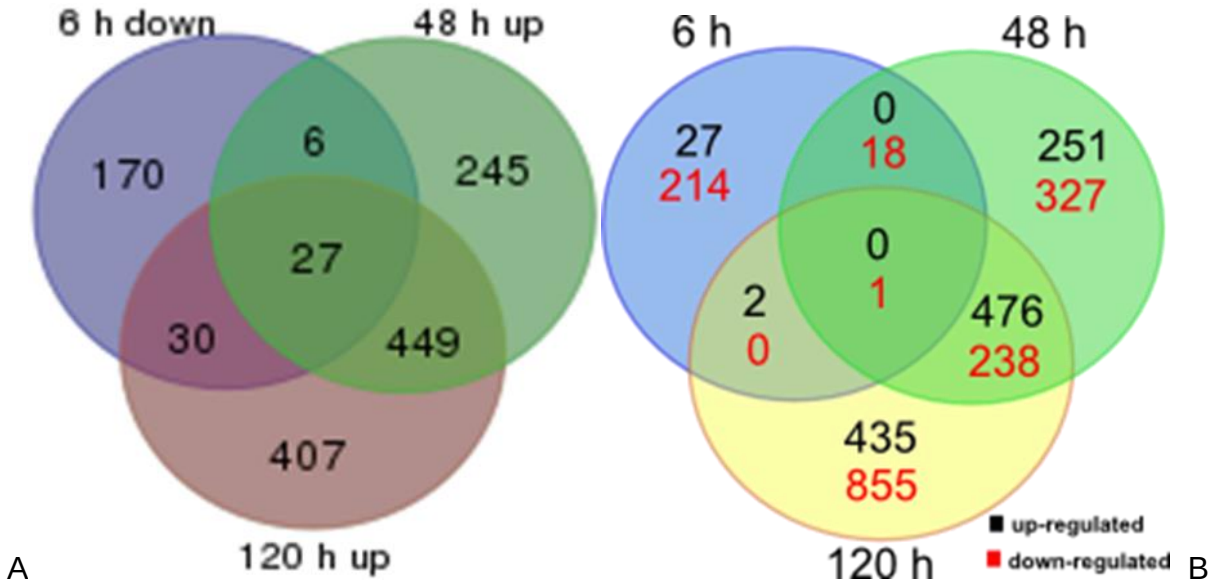


Figure 3-10. Venn diagram showing the number of up-regulated or down-regulated genes commonly modulated at 6 hpi, 48 hpi and 120 hpi following Xcc306 inoculation relative to Xcc306Δ*pthA4* inoculation. A) Number of genes down-regulated at 6 hpi (upper left) while up-regulated at 48 hpi (upper right) and 120 hpi (lower). B) Number of overlapping genes similarly up-regulated (in black font) or down-regulated (in red font) at all 6 hpi, 48 hpi and 120 hpi. The genes with fold change no less than 3 and adjust *P* value less than 0.05 were considered up- or down-regulated.

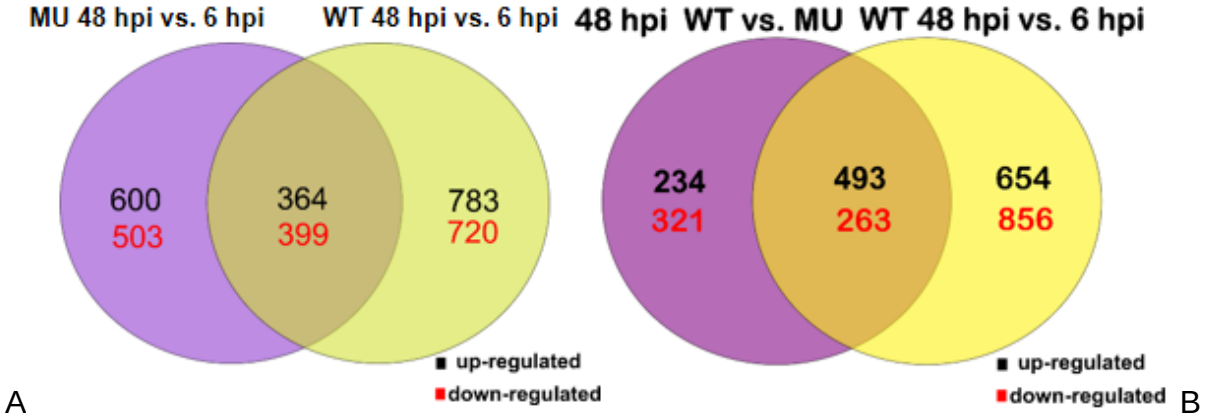


Figure 3-11. Venn diagram showing the number of overlapped DE genes between several sets of comparisons. A) Number of genes equally up-regulated (in black) or down-regulated (in red) in 48 hpi versus 6 hpi consequent to wild-type Xcc306 (right) and mutant Xcc306Δ*pthA4* (left) inoculations. B) Number of genes similarly up-regulated or down-regulated in two comparisons, WT versus MU infection at 48 hpi (left) and 48 hpi versus 6 hpi following Xcc306 infiltration (right).

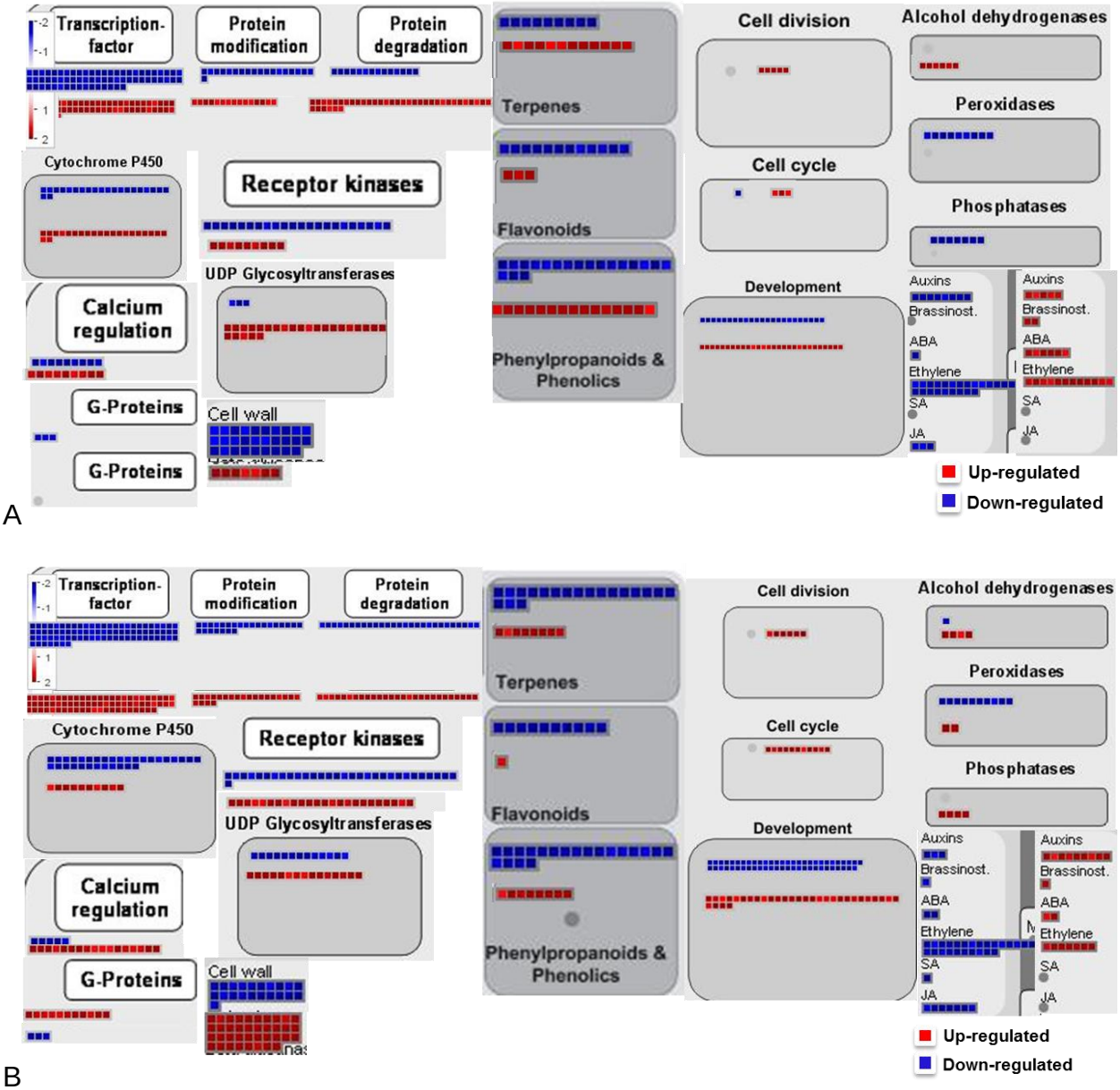


Figure 3-12. Contrastive visualization of up-regulated genes (red squares) and down-regulated genes (blue squares) in respect to 48 hpi versus 6 hpi following the challenge of both mutant Xcc306Δ*pthA4* (A) and wild-type Xcc306 (B) formed by MapMan software.

CHAPTER 4
IDENTIFICATION AND CHARACTERIZATION OF PTHA4 TARGET GENE IN CITRUS

Background Information

The mechanisms for the recruitment of type III effectors to paralyze the plant defense pathways and how these effectors are regulated in host context are far from understood yet. However, fortunately, the striking discovery of a type of transcription activator-like (TAL) effector (also TALEs) provides a relatively clear mechanism of how the effectors target and sabotage host plant, which is also the most extensively studied effectors in *Xanthomonas*.

TAL Effectors are the Pathogenicity and Avirulence Determinant

TAL effectors distribution. The genes encoding TAL effectors are members of *avrBs3/pthA* gene family, and they are predominantly present in most *Xanthomonas* genomes and in some *R. solanacearum* genomic DNA (Heuer et al., 2007; Boch and Bonas, 2010; Li et al., 2013a). After the first functional identification of *pthA* gene (Swarup et al., 1991), until now, more than 100 *AvrBs3/pthA* family members from different *Xanthomonas* species and pathovars have been identified (deposited in Genebank). For most xanthomonads, although each contains multiple copies *avrBs3/pthA* family gene, only a limited number of them have been found to be functional in contributing to major virulence determinants for the disease (Schornack et al., 2013). Also no *avrBs3/pthA* family genes have been described in some *Xanthomonas* species, such as *X. camperstris* pv. *camperstris*, *X. perforans*, and *X. camperstris* pv. *musacerum* (Moreira et al., 2004; Studholme et al., 2010; Potnis et al., 2011).

TAL effectors in *Xanthomonas citri.* For the citrus canker-causing *Xanthomonas*, most of the strains from Xcc type A have more than 3 copies of *pthA* genes, and the numbers of *pthA* gene in type A*, A^W vary from 1 to 3 copies according to the strains (Lee et al., 2008). In Xcc strain K21, four *avrBs3/pthA* family genes were identified to have very high similarity with *pthA* genes in strain 306 respectively. Among these four genes, *hssB3.0*, which is the most similar to *pthA2* in strain 306 and evolves from a recombination event among the *pthA* homologs, specifically suppressed virulence and induced a defense response in *C. grandis* (cv. Otachibana), but had no function in citrus genotype such as *C. sinensis*. A second gene *pthA-KC21*, which corresponds to *pthA4* in strain 306, had a role in eliciting hyperplastic canker symptom but not in water-soaked lesion symptom formation on citrus plants (Shiotani et al., 2007)**.** Five different *Xanthomonas* strains that cause citrus canker, A, A*, A^w, B and C, each contains only one functional *pthA* homology termed *pthA*, *pthA**, *pthAw*, *pthB*, *pthC* respectively, which confer typical canker disease symptom on citrus plants, but none determines the host range (Al-Saadi et al., 2007).

Mechanisms of TAL effectors in contributing to susceptibility and HR. The TAL effectors contribute to pathogenicity or HR various ways. Some of the TAL effectors are able to suppress plant HR defense response. When transformed with *avrBs3/pthA* genes *apl1*, *avrXa7* or *avrXa10*, *Pseudomonas fluorescens* compromised previously incited HR phenotype and the expression of tobacco HR related genes (Fujikawa et al., 2006). The TAL effector proteins sometimes also contributed to bacterial growth in the field. AvrBs3 from *X. axonopodis pv. vesicatoria* promoted the spread and enhanced fitness of the bacterium in the field (Wichmann and Bergelson,

2004). The transcription factors in planta were also demonstrated to be modulated by TAL effectors. In rice, the induction of two bZIP transcription factor genes *OsTFX1* and *OsTFIIAγ1* which mediate host susceptibility is dependent on TAL effectors PthXo6 and PthXo7, respectively (Sugio et al., 2007). The same TAL effector can also contribute to different functions in distinct plant cultivars. An AvrBs3-like effector, AvrHah1, from *X. gardneri* was characterized to enhance virulence in the pepper cultivar ECW and also at the same time had avirulence activity to cause HR in pepper *Bs3*-containing line ECW-30R (Schornack et al., 2008).

Secondary Structure Hallmarks of TAL Effectors

Generally in nature, *avrBs3/pthA* products have nearly identical leucine-rich tandem repeats of 33-35 (mostly 34) amino acids generally ranging from 12 up to 33 followed by a half repeat motif in their central portion, plus a leucine zipper region, three putative short nuclear localization signal sequences(NLS), an acidic transcriptional activation domain(AD) in the C-terminus and conserved N-terminus for T3SS secretion and translocation signals; in particular, the structure of a typical TALE, PthA4, was displayed here, which contains 17.5 direct repeats as PthA (Brunings and Gabriel, 2003) (Figure 4-1A). In most cases, the NLS and AD domains are indispensable to the activities of this type of protein (Schornack et al., 2006). The importance of NLS domains was shown by nuclear localization of fusion proteins in onion bombardment experiments and genetic studies, and was confirmed by mutagenesis experiments (Boch and Bonas, 2010). Yang *et al*. (Yang et al., 2011) acquired transgenic sweet orange lines with citrus canker resistance by transforming *pthA-nls* to the plant; this transformation interrupted the binding between NLS of PthA and the plant importin α protein, which demonstrated the importance of NLS domain in disease development.

Furthermore, deletion of the AD can render the protein inactive and fail to trigger the target genes (Szurek et al., 2001; Römer et al., 2007). Variation exists between the central repeats primarily at residues 12 and 13, which are referred to as repeat-variable diresidues (RVD); the number and arrangement of the repeat units varies between the TAL effectors, which may collectively contribute to their functional diversity, given that the N terminus and C terminus of them are highly conserved (Bogdanove et al., 2010).

TAL Effectors Directly and Specifically Recognize Plant Host Genes

In contrast to most of the effectors achieving functional redundancy by promiscuously targeting host molecules with low specificity and affinity, it has been validated that TAL effector proteins can directly and specifically interact with the promoter of plant genes, with the binding sites being designated as EBEs (Effector Binding Elements) or *UPT* box (up-regulated by TAL effectors) (Römer et al., 2007; Hann et al., 2010). Through the interaction, the TAL effectors regulate the transcription of plant susceptible or resistant genes thus causing disease or hypersensitive reaction.

Genes in plant targeted by TAL effectors. Recently, for example, it revealed that the repeat region of AvrBs3 directly bound to the promoter of a pepper transcription factor gene *upa20* and induced its expression through the activation domain (Kay et al., 2007). The expression of a rice *R* gene *Xa27* can be activated and cause HR after being challenged by bacteria harboring TAL effector AvrXa27 (Gu et al., 2005). Through microarray analyses**,** a rice dominant susceptible gene *Xa13* (also known as *Os8N3*) was identified, whose transcription was activated by TAL effector PthXo1 from *Xoo* strain PXO99 through the direct binding with UPT_{PthXo1} box (Yang et al., 2006a; Römer et al., 2010). Furthermore, one gene may be the target of several TAL effectors from multiple strains with the same or divergent EBEs in promoter. For example, *Bs3* and

upa20 in pepper are the targets of TAL effector AvrBs3 from *X. campestris* pv. *vesicatoria* and AvrHah1 from *X. gardneri*; although they have 17.5 and 13.5 repeats, they contain similar RVDs, so they may recognize common EBE sequences (Römer et al., 2007; Schornack et al., 2008). *Os11N3* in rice is recognized and activated by AvrXa7 and PthXo3 from *X. oryzae* pv. *oryzae* PXO99^{A}, while it is also the target of TalC from strain BAI3 of African *X. oryzae* pv. *oryzae* (Antony et al., 2010; Yu et al., 2011). More *UPT* genes and *UPT* boxes are being described; Kay *et al*. (Kay et al., 2009) identified another 11 new *UPA* (up-regulated by AvrBs3) genes in pepper that are induced by AvrBs3 effector, demonstrating that the *UPA* boxes are very conserved in pepper (TATATAAACCN2–3CC).

Most of the *UPT* boxes are located approximately 40-80 bp upstream of the transcription start site; these boxes are sufficient for activation**,** since their functionality is independent of promoter context (Römer et al., 2009b). For different TAL effectors, they have corresponding variable *UPT* box with very high specificities, even an AvrBs3 deletion derivative AvrBs3Δrep16, which lacks four repeats, recognized mutated *UPA* box (13 bp deletion) that cannot be recognized and activated by AvrBs3 (Kay et al., 2009). So obviously, the sequence of the EBE or UPT box depends directly and specifically on the sequence of repeats in the TAL effectors, which is defined by both the number and order of repeat units (Herbers et al., 1992).

TAL effector and DNA binding specificity. Spectacularly, Moscou and Bogdanove (Moscou and Bogdanove, 2009) and Boch *et. al* (Boch et al., 2009) cracked the code (called TALE code) of the interaction specificity between the RVD and EBE sequence both computationally and experimentally, with one RVD pairing to one

specific nucleotide in the EBE, some repeat types are specific for a particular nucleotide whereas others recognize multiple nucleotides. All of the EBEs start with a ‘T’ that precedes the first repeat (termed repeat zero); the non-RVD residues seem to have no impact or only a minor impact on the specificity. The one-one RVD and DNA base specificity is shown in Figure 4-1B, RVDs and the predicted target DNA sequence of PthA4 are exhibited in Figure 4-1A. The mutation, substitution or truncation of the *UPT* box can dramatically abolish the binding activity between TAL effectors and the promoters. Deletion of 5’ terminal ‘T’ of UPT_{PthXo6}, UPT_{AvrXa7} and UPT_{PthXo1} boxes produced significantly reduced binding activity and inducibility that was mediated by corresponding TAL effectors (Römer et al., 2010). Another experiment also showed that when mutated at the 5’-terminal second, third or fourth nucleotide of UPT_{PthXo1} box, as well as the truncation of more than 2 nucleotides in 3’ -terminal, the binding between PthXo1 and UPT_{PthXo1} was not detected (Yuan et al., 2011). The code also have lots of degeneracy, and the efficiencies of different RVDs are variable; HD and NN are strong RVDs, while NI, NG, NH and NK are weak RVDs (Streubel et al., 2012). It has been postulated that the non-optimal RVD-DNA contributes more strongly than matching RVD-DNA to the overall interaction and these non-matching combinations are length, number, position and context dependent (Scholze and Boch, 2010). For instance, deletion of the last three nucleotides of the predicted $UPT_{AvrXa27}$ box can still trigger a hypersensitive response by AvrXa27 (Römer et al., 2009a); it seems that sometimes the mismatch or omission especially in 3’-terminus may just cause the reduction but not loss in binding activity. Moreover, the TAL effector from *R. solanacearum* named RipTAL recognized DNA in similar manner as TALEs did, but its binding box is

preceded by a 5’ G rather than a 5’ T, and in contrast to TALEs, its non-RVD residues also considerably affects the DNA binding activity (de Lange et al., 2013). So detecting the crystal structure of the TAL effector and the model of TALE-EBE interaction is necessary.

Crystal structures of TALE-DNA binding. It has been predicted that the AvrBs3 repeat domain structurally resembled tetratrico peptide repeat (TPR) protein which is a structural motif consisting of 3–16 tandem repeats of 34-aa residues and mediates protein–protein interactions as well as assembly of multi-protein complexes; they also showed high similarity to pentatrico peptide repeat (PPR) proteins, which carry 2-26 degenerated tandem 35-aa repeats or alternating 31-aa,36-aa repeats and mediate RNA binding; each repeat unit of AvrBs3 is predicted to form two α-helices (Schornack et al., 2006; Murakami et al., 2010). Recently, the crystal structure of PthXo1 bound to its DNA target was determined using high-throughput computational structure prediction and validated by heavy-atom derivatization; each repeat forms a left-handed, two α-helices bundle that exposes an RVD-containing loop to the DNA and the repeats self-associate to form a right-handed superhelix wound around the DNA major groove (Mak et al., 2012). By testing a 15.5 repeat TALE, another group also reported the similar crystal structures with 12th residue stabilizing the RVD loop, and the 13th residue contacting with a specific nucleotide base (Deng et al., 2012). But the overall tertiary structure of the TALE-DNA complex remains to be further determined.

Traits of TAL Effector Target Genes in Plants

As for the known plant host genes targeted by TAL effectors, in different plants and recognized by distinct TAL effectors, the targets have diverse functions in plant physiology and development other than susceptibility or resistance. The characterized

TAL effector targets are listed in Table 4-1. For the *R* genes that are targeted and activated by TAL effectors, so-called executor *R* gene, such as *Bs3* from pepper and *Xa27* from rice, they are distinct from most of the *R* genes which are transcribed constitutively and encode NB-LRR proteins; only one NBS-LRR protein, tomato Bs4, was reported to recognize TAL effectors such as AvrBs4 but is independent of DNA binding activity (Schornack et al., 2004; Gu et al., 2005; Römer et al., 2007). In pepper, the *R* gene *Bs3* and *Bs3-E* that is recognized and mediated by AvrBs3 and AvrBs3Δrep16 respectively, encode flavin monooxygenases (Römer et al., 2007); another direct target of AvrBs3, so-called *upa20*, encodes a bHLH family transcriptional factor that contains a basic helix-loop-helix domain, which acts as a regulator of cell enlargement (Kay et al., 2007). In rice, two susceptible (*S*) genes, *OsSWEET11*/*Os8N3*, target of PthXo1, and *OsSWEET14/Os11N3*, target of AvrXa7, both belong to *MtN3* gene family that encodes nodulin-related proteins, which mediate sugar efflux to feed bacterial and promote the growth of the bacterial (Yang et al., 2006a; Antony et al., 2010; Chen et al., 2010); a second study also revealed that *OsSWEET11* was a copper transporter to remove copper from xylem vessels and facilitate multiplication and spread of *Xoo* (Yuan et al., 2010); another rice *S* gene with minor effects on susceptibility, *TFX1*, which is recognized and activated by Xoo TAL effector PthXo6, is a member of the basic region leucine zipper (bZIP) transcription factor family gene, while *OsTFIIAγ1* that is highly induced by PthXo7, is a small subunit of the transcription factor IIA (Sugio et al., 2007). In addition, a gene in pepper named *upa16* that is the paralog of *MtN3* family, was also induced by AvrBs3 (Kay et al., 2009); and one more rice *MtN3* family gene member *xa25*, recessively mediated race-specific resistance to Xoo strain

PXO339 (Liu et al., 2011); these results demonstrate that the *MtN3* family genes may be widely involved in TAL effectors targeting and plant host-pathogen interactions for nutritional supply.

Target Discovery and Gene Engineering by Exploiting Features of TALE

The elucidation of the TALE code expedited further discovery of TAL effector target genes and opens the door to biological and biotechnological application by using artificially designed TALEs or EBEs (Bogdanove et al., 2010; Scholze and Boch, 2011; Doyle et al., 2013; Muñoz Bodnar et al., 2013; Schornack et al., 2013).

Target prediction. The TAL effector gene targets now can be identified through integration of transcriptional and computational approaches. Several TAL effector target prediction tools have been established for easy deducting the presence of EBEs in gene promoter from RVD sequences in TAL effectors (Doyle et al., 2012; Grau et al., 2013). (https://tale-nt.cac.cornell.edu/node/add/talef-off, http://galaxy2.informatik.uni-halle.de:8976/). Romer *et al*. (Römer et al., 2010) successfully predicted and validated potential *UPT* boxes by employing the TALE code; the *UPT* boxes from the rice genes *Xa13*, *OsTFX1*, *Os11N3* were identified to be targeted directly and specifically by the *X. oryzae* pv. *oryzae* TAL effectors PthXo1, PthXo6, AvrXa7, respectively. In a proof-of-principle experiment, the resistance gene *Bs4C* as well as the EBE were identified from pepper in the presence of AvrBs4 by jointly using RNA-seq and the TALE code (Strauß et al., 2012), which was distinct from *Bs4* in tomato; it also paves a less laborious avenue for isolating *R* genes from crop plants and to avoid the painstaking positional cloning.

Transcriptional control in plant. After investigating specificity in the interaction between different TAL effectors and *UPT* boxes of plant *R* genes, it is possible to

engineer *R* genes to contain multiple *UPT* boxes in promoter and recognize diverse TAL effectors, thus producing a promoter trap and gaining the potential to generate board spectrum and durable resistance. Romer *et al*. (Römer et al., 2009a) demonstrated that when three functionality and sequence distinct *UPT* boxes UPT_{Xa27}, UPT_{AvrBs3}, and $UPT_{AvrBS3\Delta rep16}$ were combined into one complex promoter, it could recognize all three TAL effectors and trigger HR. By stacking six EBEs to the rice *Xa27* gene, the executor *R* gene specificity was broaden to resistant to more diverse strains of *X. oryzae* (Hummel et al., 2012). Morbitzer *et al*. (Morbitzer et al., 2010) used the TALE code in an opposite manner by designing TAL effectors that could activate the defined chromosomal genes. They generated a special TAL effector that matched against a pre-determined sequence in tomato *Bs4* gene promoter through the code when using AvrBs3 as a structural scaffold except for the RDV residues; this custom TAL effector was able to specifically target and activate the *Bs4* gene expression both through co-delivery into *N. benthamiana* leaves and endogenous activation in tomato. They also created two TAL effectors to target the promoters of two *A. thaliana* genes *EGL3* and *KNAT1*, which acted as transcriptional activators of the endogenous genes *in planta*.

Application in mammalian and genomic editing. Due to the clear and simplicity of TALE code, the TAL effectors have also been designed to recognize the target DNA sequences predicted by the TALE code in zebrafish, yeast and mammalian cells. TAL effectors have been artificially designed for transcriptional manipulation of the genes from the mammalian genome when fusing the mammalian activation domain to TAL effector DNA binding domains (Miller et al., 2011; Zhang et al., 2011). In these two years, emerging papers in technology and application of genome engineering have

been published on TALEN (TALE nuclease) that are created by tethering FOK I endonuclease domain into TAL effectors and results in efficient protein cleavage. Some most recent and excellent papers in this aspect are listed here (Sanjana et al., 2012; Gaj et al., 2013; Kim et al., 2013; Li and Yang, 2013; Schmid-Burgk et al., 2013; Sun and Zhao, 2013).

Results

Experiments Outline

In this study, we elucidated the function of TAL effector PthA4 from Xcc as a pathogenicity contributor. Here, the candidate PthA4 target genes which may also behave as *S* gene were selected from the PthA4-dependent transcription profiles described in Chapter 2. The selection was based on three considerations, 1) high fold increase in expressions at 48 hpi and 120 hpi with respect to WT versus MU; 2) presence of a candidate EBE predicted from RVDs of PthA4 in the promoter regions (Figure 4-1); 3) relatedness to known *S* genes and known TALE targets. The candidate genes were validated and also subjected to complementation by artificially designed TAL effectors (dTALes) that were either optimized by the consensus TAL effector binding codes or targeted to novel promoter sequences. Afterwards, another four different *pthA4* homologies that determine pustule formation on citrus, *pthAw, pthA*, pthB and pthC*, each of whom has different and unique repetitive central domain, were also tested for binding and activation abilities to the candidate genes.

CsLOB1 and *CsN3-1* are Candidate Targets of TAL Effectors PthA4

From the microarray data, the genes showing significant higher (adjust $P<0.01$) expression levels in tissue infiltrated with wild-type Xcc306 in comparison to tissue infiltrated with Xcc306Δ*pthA4* were selected as potential candidate host *S* genes. The

top 30 most highly up-regulated genes were selected and the promoters were scanned for probable PthA4 binding elements (Table 4-2). One gene that represented by two probesets was identified. In addition, a gene encoding member of MtN3 family was found to be highly induced in another microarray experiment conducted by Zhang et al. from Dr. Frank White's lab in Kansa State University (data not shown), which also contains a candidate EBE_{PthA4}. These two genes were characterized further, although the latter one did not appear in the top up-regulated gene sets from our microarray data.

The gene represented by probes Cit.37210.1.S1_at and Cit.35190.1.S1_at contained sequence very close to the canonical PthA4 binding element (EBE_{PthA4}), which is located 92 bp upstream of predicted transcription start site basing on EST sequences. This gene encodes a member of the lateral organ boundaries (LOB) domain family of transcription factors and was designated *CsLOB1*. The most closely related homologs are AtLBD1 and AtLBD11 of *Arabidopsis* (Figure 4-2). Another gene, which is represented by Cit.3027.1.S1_s_at, contains a sequence close to canonical EBE_{PthA4} which starts 43 bp upstream of the potential transcription start site. It represents a homolog to the TAL effectors targeted *S* genes *OsSWEET11* and *OsSWEET14* in rice, and was designated as *CsN3-1.* The expression of both genes was observed to be elevated as determined by qRT-PCR analysis of mRNA from tissue infected either with Xcc306 in comparison to mRNA from tissue infected with Xcc306Δ*pthA4*. A time course of 12 h, 24 h, and 48 h after inoculation in sweet orange indicated that expression of both genes reached high levels by 24 hours after the infiltration (Figure 4-3).

CsLOB1 and *CsN3-1* Promoters Direct TAL Effector-Dependent Expression

The respective promoters of *CsN3-1* and *CsLOB1* were fused to a *uidA* (β-glucuronidase, GUS) reporter gene and expressed transiently by *Agrobacterium*-

mediated transfer. Truncated promoters and altered versions in the predicted EBEs were also tested in co-inoculation assays with Xcc306 in citrus leaves (Figure 4-4). The wild-type promoter fragment of *CsN3-1* directed GUS activity when co-inoculated with the wild-type strain Xcc306 and the complemented strain Xcc306Δ*pthA4:pthA4*, while no GUS activity was detected when co-infiltrated with strain Xcc306Δ*pthA4* (Figure 4-5A; CsN3Pwt). Co-inoculations with the truncated, substituted, and deleted versions of *CsN3-1* promoter and Xcc306 resulted in little to no GUS activity (Figure 4-5A, CsN3PT, CsN3PM1, and CsN3PD, respectively). At the same time, wild-type, truncated and substituted versions of *CsLOB1* promoter were activated at the same level by Xcc306 (Figure 4-4 and 4-5A, CsLOBPT and CsLOBPM1). The deletion within the predicted EBE and TATA box of *CsLOB1* (Figure 4-5A, CsLOBPD) resulted in the loss of PthA4-mediated expression (Figure 4-5A). As a control, the promoter of another highly up-regulated gene Cit.7877.1.S1_at was not able to be stimulated by PthA4 (Table 4-2; Figure 4-5A*,* column C).

The results for the alterations to changed *CsN3-1* and *CsLOB1* promoters indicated that, though remarkably similar, the promoters of the respective genes with respect to the candidate EBEs have minor but important differences. The candidate EBE_{PthA4} for *CsLOB1* is thought to be contained within the region of the TATA box, based on the results with the truncated version CsLOBPT (Figure 4-4, construct 8). Additional base substitutions and insertions were created within the truncated version of *CsLOB1* and tested to further corroborate the function of this region as an EBE_{Pth4} (Figure 4-4, constructs 8-12). Promoter variant CsLOBPM3, which has a substitution of GG for CC at 8th and 9th positions in the EBE had a severe effect on PthA4-dependent

promoter activity, while the substitution of T (CsLOBPM2) for C only at position 8 had little effect on activity (Figure 4-5B, constructs 9-11). A single nucleotide insertion at position 11 (CsLOBPins) in EBE_{PthA4} resulted in loss of GUS activity (Figure 4-5B).

The *Agrobacterium tumefaciens*–mediated transient ectopic expression of *pthA4* was also able to activate the same *CsLOB1* or *CsN3-1* promoter patterns in *N. benthamiana*, respectively (Figure 4-6).

Artificial dTALes Targeting *CsLOB1* Induce Pustule Formation

Artificial dTALe genes with *pthA4* as a backbone sequence were designed with repeats specifically targeting unique sequences within promoters of *CsN3-1* and *CsLOB1*, respectively, using optimized RVD residues. The genes were designated *dCsLOB1.1*, *dCsLOB1.2*, *dCsN3-1.1* and *dCsN3-1.2*, introduced into Xcc306Δ*pthA4*, and tested for activity on citrus leaves. Among them, *dCsLOB1.1* and *dCsN3-1.1* were designed and synthesized by Dr. Bing Yang's lab in Iowa State University, while *dCsLOB1.2* and *dCsN3-1.2* were created by Dr. Nian Wang's lab at University of Florida. Their RVDs in the repeats, sequences and locations of the targeting DNA were shown in Figure 4-7. The validity of these dTALes were first assessed by qRT-PCR and GUS assay. Xcc306Δ*pthA4* with either *dCsLOB1.1* or *dCsLOB1.2* induced *CsLOB1* expression, but did not induce *CsN3-1*, while Xcc306Δ*pthA4* with *dCsN3-1.1 or dCsN3-1.2* induced the expression of *CsN3-1* but not *CsLOB1* expression (Figure 4-8A). In parallel, the Xcc306Δ*pthA4* with the individual dTALe genes were tested for the ability to induce the promoter-*uidA* reporter genes by quantitative transient GUS assays in sweet orange, only *dCsLOB1.1* and *dCsN3-1.1* were correctly examined by us. Xcc306Δ*pthA4* harboring *dCsLOB1.1* directed expression of *CsLOB1* promoter, but not *CsN3-1* promoter, and, conversely, Xcc306Δ*pthA4* harboring *dCsN3-1.1* drove expression from

the *CsN3-1* promoter and not the *CsLOB1* promoter fusions in citrus leaves (Figure 4-8B). The dTALe-complemented strains of Xcc306Δ*pthA4* were infiltrated into sweet orange to determine what effect the artificial effectors would have on the disease phenotype. Only inoculations strains of Xcc306Δ*pthA4* with either dTALe targeting *CsLOB1* resulted in pustule formation (Figure 4-9A and B), although in low inoculum concentration (5×10^5 cfu/ml), the symptom was not fully restored as PthA4 did (Figure 4-9C). In addition, in cell density assay, the bacterial leaf populations were significantly higher in sweet orange leaves inoculated with Xcc306Δ*pthA4:dCsLOB1.1* when compared to Xcc306Δ*pthA4* but lower than Xcc306Δ*pthA4*:*pthA4* by 9 dpi (Figure 4-10).

CsLOB1 is Target of Alternate TAL Effectors Involved in Citrus Canker

The TAL effectors genes *pthAw*, *pthA**, *pthB*, and *pthC*, which were previously shown to be associated with pustule formation (Al-Saadi et al., 2007), were tested for the ability to induce pustule formation in Xcc306Δ*pthA4*. The four genes, similar to *pthA4*, could confer pustule formation in Xcc306Δ*pthA4* (Figure 4-11). And the complementing strains of Xcc306Δ*pthA4* with each respective gene were inoculated on three hosts, sweet orange, grapefruit, and key lime to assess the ability to induce *CsLOB1* and *CsN3-1*. PthA4, PthAw and PthA* led to induction of both *CsN3-1* and *CsLOB1* in the three species, while PthB and PthC could only direct the expression of *CsLOB1*, but not *CsN3-1*, in all three species (Figure 4-12). Bacterial leaf populations of Xcc306Δ*pthA4:pthB* in sweet orange were the same as Xcc306Δ*pthA4:pthA4*, and higher than the mutant Xcc306Δ*pthA4* by nine days after infiltration (Figure 4-13). The predicted EBEs of PthB and PthC were supposed to be the same and located six bases upstream of EBE_{PthA4}, and the predicted EBEs of PthAw and PthA* appeared to be located at the same position as that of PthA4 (Figure 4-14A). Xcc306Δ*pthA4:pthB* and

Xcc306Δ*pthA4:pthC* strains could only direct expression of the wild-type *CsLOB-1* promoter but not the truncated versions, which are missing three bases of the predicted EBEs for PthB and PthC, PthB and PthC also did not activate the *CsN3-1* promoter fused reporter gene(Figure 4-4 and 3-14B). Moreover, the inducibility feature of PthAw was also inspected in *N. benthamiana* through *Agrobacterium*–mediated transient ectopic expression of *pthAw* (35S drove), it revealed that the pattern was analogous to that by PthA4 shown in Figure 4-6 (Figure 4-15).

Materials and Methods

PthA4 Target Gene Search

Thirty top highest up-regulated genes by Xcc306 relative to Xcc306Δ*pthA4* at 48 hpi from microarray described in Chapter 3 were selected. The 1000 bp upstream of CDS (coding DNA sequences) of these gene were obtained from phytozome (http://www.phytozome.org/citrus.php), and the regions were scanned by Target Finder using RVD sequence of PthA4 (Doyle et al., 2012).

GUS Reporter and Gene Overexpression Construction

Sequences about 600 bp upstream of *CsN3-1* and *CsLOB1* CDS were amplified from sweet orange genome DNA, promoter derivative fragments were obtained by amplification with different primers (listed in Table 2-2). The promoter fragments were digested with BamH I and Hind III to fuse with *uidA* gene in pBI101 vector. The constructions were transformed into *Agrobacterium* strain EHA101. To overexpress *pthA4* and *pthAw*, the amplified genes were inserted after 35S promoter and before 35S terminator in vector pUC118/35S polylinker when digested with *Apa* I and *Xho* I, then these constructions were cut with *Hind* III and *Xba* I to ligate into pCAMBIA2200, the constructions were introduced into *Agrobacterium* strain LBA4404.

β-Glucuronidase (GUS) Assays

For transient GUS expression in citrus plant, *A. tumefaciens* was cultivated at 28 °C in YEP plates, and the *Agrobacterium* suspended in solution containing 10 mM MgCl2, 10 mM MES (pH 5.6) and 100 uM acetosyringone with concentration OD600 = 0.8 was infiltrated into sweet orange, after 5 hours, the *Xanthomonas* with concentration of OD600 0.3 was infiltrated at the same area. After 5 days, the GUS activities were measured (Figueiredo et al., 2011b). For the transient expression in *Nicotiana benthamiana*, two *Agrobacterium* suspensions were mixed at ratio of 1:1, and the GUS assay was preformed 3 days after infiltration. For quantitative GUS assay, one leaf disc (1 cm diameter) was grounded with 400 µl GUS extraction buffer (50 mM $NaPO_4$ (PH 7.0), 1 mM Na_2EDTA, 0.1% SDS, 0.1% Triton X-100, 10 mM DTT). After spin at 4 °C for 15 min in top speed, 25 µl of the supernatant was mixed with 225 µl GUS assay buffer (GUS extraction buffer supplied with 0.44 mg/ml 4-methyl umbelliferyl β-D-glucuronide), and kept in 37 °C for 1 hour, the reaction was stopped with 0.2 M Na_2CO_3. Measurement was done in a plate reader (CytoFluor II) at 360 nm (excitation) and 460 nm (emission) with 4-methyl-umbelliferon (MU) dilutions as standard. The protein quantification was performed by Bradford assay (BioRad, Hercules, CA). For qualitative GUS assay, one fresh leaf disc was put into GUS staining buffer (50 mM $NaPO_4$ PH 7.0, 0.1% Triton X-100, 10 mM EDTA, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 0.5 mg/ml X-gluc) and incubated at 37 °C overnight, the discs were destained in ethanol.

Bacterial growth assay and qRT-PCR processes were described in previous Chapters.

Discussion

CsLOB1 is a Citrus Susceptibility Gene Targeted by Diverse TAL Effectors

In this study, we demonstrated that pustule formation of citrus bacterial canker results from the co-option of a single host *S* gene *CsLOB1* via PthA4 and its homologs. Although PthA was not tested specifically, the effector has almost the same predicted target site as PthA4 (Boch et al., 2009). Recently, a gene encoding LOB domain protein was also computationally predicted to be the most promising target of PthA4 through a promoterome-wide search (Grau et al., 2013). Alternate TAL effectors PthAw, PthA*, PthB, PthC from genetically diverse *Xanthomonas* strains that cause citrus canker were able to restore the ability to produce typical pustules to the impaired Xcc306Δ*pthA4* strain; through qRT-PCR, all four PthA4 variants were also determined to induce and target the same gene (Figure 4-12). Citrus canker occurs across a variety of citrus species, and *CsLOB1* is present and activated by TAL effectors in sweet orange, grapefruit and Mexican lime (Figure 4-12); the obvious differential activation levels may attribute to minor sequence changes in the promoter EBE region. All the variants of PthA and the particular species were compatible with *CsLOB1* induction and pustule formation, possibly reflecting that the genes have converged to the same target. To further substantiate this conclusion, dTALes targeted to unique or optimal binding sites in promoter region were constructed and found to induce *CsLOB1* and restore pustule formation when expressed in bacteria (Figure 4-9B). In *gus* reporter assay, the variants of PthA except PthA* strongly drove the *CsLOB1* promoter expression (Figure 3-14B), which also supported the hypothesis that *CsLOB1* was targeted and activated by these TAL effectors. The poor activation by PthA* may be accounted for by the low specificity thus low affinity of EBE with *CsLOB1* promoter (5 bases mismatch, Figure 4-14A);

according to the symptoms, PthA* also did not fully restore pustule formation to normal phenotype (Figure 4-11); but interestingly, PthA* induced *CsLOB1* expression at almost the same level as others did (Figure 4-12). Although no evidence was obtained in this study, the *CsLOB1* gene may represent adaptations to avoid or compensate for host induced resistance genes or changes in *CsLOB1* promoter sequences in as yet unidentified species or cultivars of citrus, just as *Os8N3*, which is the allele of recessive *R* gene *xa13* and only differs in EBE_{PthXo1} region (Yang et al., 2006a).

The predicted EBEs in *CsLOB1* promoter match the various TAL effectors and meet the general prediction requirements of known EBEs targeted by other TALes, and, for the most part, the results of experimental tests of EBE function for *CsLOB1* were consistent with predictions. The elements are located approximately 100 bp upstream of 5' UTR and overlapped with the presumed TATA box as previously indicated for the typical EBEs (Grau et al., 2013). PthA4, PthAw, and PthA* share the same predicted EBE in *CsLOB1* promoter, although the respective RVDs differ (Figure 4-14A). The truncated version of the target site, which eliminated the natural upstream sequences in *CsLOB1*, was functional both in citrus and *Nicotiana* (Figure 4-5 and 4-6). Changes in the respective TATAA boxes for *CsLOB1* and *CsN3-1* eliminated expression (Figure 4-4, CsLOBPD), as might be expected for the predicted TATAA boxes for each gene. The substitution in the proximal 5' half of the binding site (CC) had severe effects for PthA4-mediated expression compared to changes in the distal sequence of TTT (CsLOBPM3 and CsLOBPM1); considering only slightly reduced activity by replacing C to T at the same position (CsLOBPM2), the damage of the TATAA box by the replacements for both CsLOBPM2 and CsLOBPM3 were exclude; and two bases substitution in extreme

5’-terminal also abolished the expression (CsLOBPM5). Furthermore, a single base insertion, which throws the distal part out of register, also eliminated effector-mediated expression for *CsLOB1* (CsLOBPins, Figure 4-4). The start of the predicted EBEs for PthB and PthC is 6 bp upstream of the EBE_{PthA4} and is similar to the *Os11N3* promoter in rice in which EBE_{PthXo3} is 2 bp in front of EBE_{AvrXa7} (Antony et al., 2010). In addition to the loss of function due to the truncation of the promoter after the start of the predicted sites for PthB and PthC, a single base change (T to C) in the second nucleotide of *CsN3-1* candidate $EBE_{PthB/C}$ entirely disrupted the induction of reporter gene (Figure 4-14). These jointly suggested that 5’-terminal part of EBE played pivotal role in binding and activating, which was in agreement with what was reported previously (Yuan et al., 2011).

Functional Characteristics of LOB Domain Family and *CsLOB1*

The lateral organ boundaries (LOB) proteins are plant specific and they are defined as containing LOB domain, which is composed of a conserved Cys repeat motif ($CX_2CX_6CX_3C$) required for DNA-binding, an invariant glycine residue, and a coiled-coil Leu-zipper-like motif ($LX_6LX_3LX_6L$) that functions in protein-protein interactions (Shuai et al., 2002). Based on their nuclear localization, the LOB proteins constitute a class of transcription factors, which recognize a 6-bp consensus DNA motif and are capable of interacting with the basic helix-loop-helix (bHLH) family proteins bHLH048 (Husbands et al., 2007). One of the targets of TAL effector AvrBs3 in pepper is *upa20*, an auxin-responsive gene and encodes bHLH family protein (Kay et al., 2007); the interaction between CsLOB1 and CsbHLH may be an important mechanism in disease development. Previous studies revealed that LOB domain proteins are involved in the regulation of plant lateral organ development, anthocyanin and nitrogen metabolism and

are responsive to phytohormones and environmental stimuli such as auxin, cytokinin, gibberellin, brassinosteroid and salinity or glucose (Majer and Hochholdinger, 2011; Gendron et al., 2012). One member of the LOB domain family, AtLBD18, was reported to bind with the promoter of *EXPANSIN14*, a gene involved in cell wall loosening (Kim and Lee, 2013). Recently, an *Arabidopsis* LOB family protein LBD20 was identified as a susceptibility gene in response to fungal pathogen *Fusarium oxysporum* and functioned in the jasmonate signaling pathway (Thatcher et al., 2012b). *LBD20* is induced by *F. oxysporum,* and overexpression of *LBD20* was correlated with increased susceptibility to *F. oxysporum* and reduced the expression of JA-regulated genes *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) and *THIONIN2.1* (*Thi2.1*). Other LOB domain family genes were also detected as being responsive to fungal and root pathogens from public *Arabidopsis* array data (Thatcher et al., 2012a).

Based on the transcription profile results, a high proportion of host genes in which expression is associated with *CsLOB1* expression during citrus canker disease are genes associated with cell wall metabolism (Figure 4-16A). We postulated that *CsLOB1* expression was associated with expression of cell wall related enzyme genes. In order to verify this hypothesis, a select group of induced genes related to expansion and wall metabolism were chosen from the most highly induced genes during infection by Xcc306 and tested for induction by qRT-PCR in the presence of dCsLOB1.1, which induced *CsLOB1*. Genes for pectic lyase, extension, α-expansin, and cellulose were found to be up-regulated in sweet orange in a similar pattern as *CsLOB1* expression (Figure 4-16B). So *CsLOB1* expression is related to numerous cell wall related proteins indicating a function in cell expansion in some otherwise normal developmental

process. To gain further insight into the roles of *CsLOB1*, more *in vivo* or *in vitro* experiments need to be conducted for confirming its binding with TAL effectors or other plant protein; transgenic citrus lines that over-express and silence this gene need to be generated; another major task is to identify downstream targets of CsLOB1 protein with the help of the consensus *cis*-element.

Possible Roles of *CsN3-1* and More Genes That May Be Involved in TALE Targeting

The results for the candidate EBEs in *CsN3-1* were more complex; no evidence was found supporting a function for the SWEET gene *CsN3-1* in citrus canker. *CsN3-1* showed TAL effector dependent expression for PthA4, PthAw, and PthA*. Only the longer promoter construct supported TALE-mediated induction. Truncation of the *CsN3-1* promoter to include only the TATAA box region and distal portion could not support expression either in citrus or *Nicotiana*. At the same time, a similar change in the distal TTT sequence of EBE site A also resulted in loss of expression in transient assays (Figure 4-5 and 4-6). However, the naturally occurring effectors PthB and PthC induce *CsLOB1* and not *CsN3-1*, and pustule development and enhanced bacterial leaf populations were only observed with dTALes targeting *CsLOB1*. Binding to the *CsN3-1* promoter may, therefore, be weak, and expression may be due to multiple binding sites including ones upstream or more complex interactions. In evolutionary terms, *CsN3-1* falls into Clade I in the phylogenetic tree as classified by Chen et al. (Chen et al., 2010), differing from the disease susceptibility genes *SWEET11&14* of rice which are in Clade III. This may indicate that the function of *CsN3-1* is independent of sucrose transport but more related to glucose transport.

Nonetheless, on the one hand, full complementation of bacterial growth and pustule formation following inoculation with low inoculum concentrations in citrus leaves was not attained with the dTALe *dCsLOB1.1* despite the apparent robust expression of *CsLOB1* (Figure 4-10 and 4-9B). The question remains as to whether expression of *CsN3-1* or other host genes represent a more complex virulence adaptation on the part of the bacterium or TAL effector mediated expression of *CsN3-1* is inconsequential. The naturally derived effector PthB did complement both pustule formation and bacterial growth lending support to the hypothesis that *CsN3-1* is not involved in citrus canker. Further analysis would be required to determine if, possibly, another member of the SWEET family is induced by PthB. In bacterial blight or rice, a yet uncharacterized SWEET gene conferred susceptibility to the Xoo strains harboring the corresponding dTALes (Li et al., 2013b). Here, the possibility exists that a single effector may have evolved to capture command of multiple host genes for optimal pathogenicity. However, further understanding of dTALe construction and natural TAL effector function will be required to draw stronger conclusions.

Table 4-1. Known TAL effector targets in various plants and their characteristics

TAL effector	Plant target	Characteristic	Description	Reference
Xcv AvrBs3	*Bs3* (pepper)	*R* gene, flavin monooxygenases	Common *UPA* box	(Römer et al., 2007)
	upa20 (pepper)	*bHLH* TF family		(Kay et al., 2007)
	upa16 (pepper)	*MtN3* family		(Kay et al., 2009)
Xcv AvrBs3 Δrep16	*Bs3-E* (pepper)	Bs3 derivative with 13 bp deletion at *UPA* box		(Kay et al., 2009)
Xg AvrHah1	*Bs3* (pepper)	*R* gene, flavin monooxygenases	Probably same EBE as *Bs3* and *upa20*	(Schornack et al., 2008)
	upa20 (pepper)	*bHLH* TF family		(Schornack et al., 2008)
Xoo PthXo1	*xa13* (rice)	Recessive *R* gene	Differ only in promoter region	(Chu et al., 2004)
	Os8N3 or *OsSWEET11*(rice)	*S* gene, *MtN3* family, sugar transport		(Yang et al., 2006a)
Xoo AvrXa7	*Os11N3* or OsSWEET14 (rice)	*S* gene, *MtN3* family, sugar transport		(Antony et al., 2010)
Xoo PthXo3				(Antony et al., 2010)
Xoo TalC				(Chen et al., 2010)
Xoo AvrXa27	*Xa27* (rice)	*R* gene, no homology		(Gu et al., 2005)
Xoo PthXo6	*OsTFX1* (rice)	bZIP TF family		(Sugio et al., 2007)
Xoo PthXo7	*OsTFIIAγ1* (rice)	Subunit of transcription factor IIA		(Sugio et al., 2007)
Xcv AvrBs4	*Bs4* (tomato)	NBS-LRR *R* gene	Non-nuclear localization	(Schornack et al., 2004)
	Bs4C (pepper)	*R* gene, no homology		(Strauß et al., 2012)

Xcv, Xanthomonas campestris pv. vesicatoria; Xg, Xanthomonas gardneri; Xoo, Xanthomonas oryzae pv. oryzae. TF, transcription factor. EBE, effector binding element; UPA, up-regulated by AvrBs3

Table 4-2. Thirty most highly up-regulated genes in sweet orange by PthA4-mediated infection

Probe ID	Log2 FC	DNA	EBE_{PthA4}	Annotation
Cit.39387.1.S1_at	7.874	N/A	No	Pectate lyase
Cit.7877.1.S1_at	7.849	CX667721	No	Cytokinin induced message
Cit.5370.1.S1_s_at	7.719	CX642883	No	pectin methylesterase inhibitor family
Cit.20041.1.S1_at	7.709	CB250345	No	N/A
Cit.2392.1.S1_at	7.251	CF831790	No	Acidic cellulase
Cit.28626.1.S1_s_at	7.124	CV710534	No	Beta expansin 6
Cit.3554.1.S1_s_at	7.016	CX663293	No	Cellulase
Cit.35754.1.S1_at	6.682	CB250305	No	Polygalacturonase-like protein
Cit.12550.1.S1_at	6.615	CD574246	No	Arabinogalactan-protein
Cit.37210.1.S1_at	6.592	BQ623314	YES	Lateral organ boundaries (LOB) domain family
Cit.20509.1.S1_at	6.56	CX644808	No	Probable pectate lyase P18 precursor
Cit.29007.1.S1_at	6.528	CX637545	No	Gibberellin-regulated protein GASA5 precursor
Cit.24058.1.S1_s_at	6.436	CD575611	No	N/A
Cit.35768.1.S1_s_at	6.346	CB250380	No	GASA5-like protein
Cit.14250.1.S1_at	6.325	CV713259	No	Acid phosphatase
Cit.11144.1.S1_s_at	6.316	CX053912	No	Cytosolic factor family protein
Cit.9528.1.S1_x_at	6.304	CX641267	No	Beta-expansin 2 precursor
Cit.17853.1.S1_s_at	6.17	CK933446	No	Pectate lyase family protein
Cit.11147.1.S1_s_at	6.053	CX674752	No	Cytosolic factor-like protein
Cit.30858.1.S1_at	6.047	CF505371	No	Expansin
Cit.25433.1.S1_s_at	6.006	CX665604	No	N/A
Cit.32260.1.S1_at	5.985	CX289947	No	Ubiquitin-like protein
Cit.35190.1.S1_at [1]	5.875	CK932995	Yes	LOB domain-containing protein
Cit.19605.1.S1_at	5.842	CK939040	No	Calcium ATPase
Cit.2949.1.S1_s_at	5.819	CN182557	No	Xyloglucan endo-1,4-beta-D-glucanase
Cit.17852.1.S1_s_at	5.809	CX294019	No	N/A
Cit.14005.1.S1_at	5.775	CV710432	No	Expansin
Cit.10482.1.S1_s_at	5.762	CX664720	No	N/A
Cit.6493.1.S1_at	5.756	CX069721	No	N/A
Cit.3027.1.S1_s_at [2]	2.504	CX048987	Yes	Nodulin MtN3 family protein

[1] represents the same gene as Cit.37210.1.S1_at; [2] not in the top 30 most up-regulated gene sets, but contains EBE_{PthA4}. FC, expression fold change at 48 hpi in comparison of Xcc306 infection and Xcc306Δ*pthA4* infection.

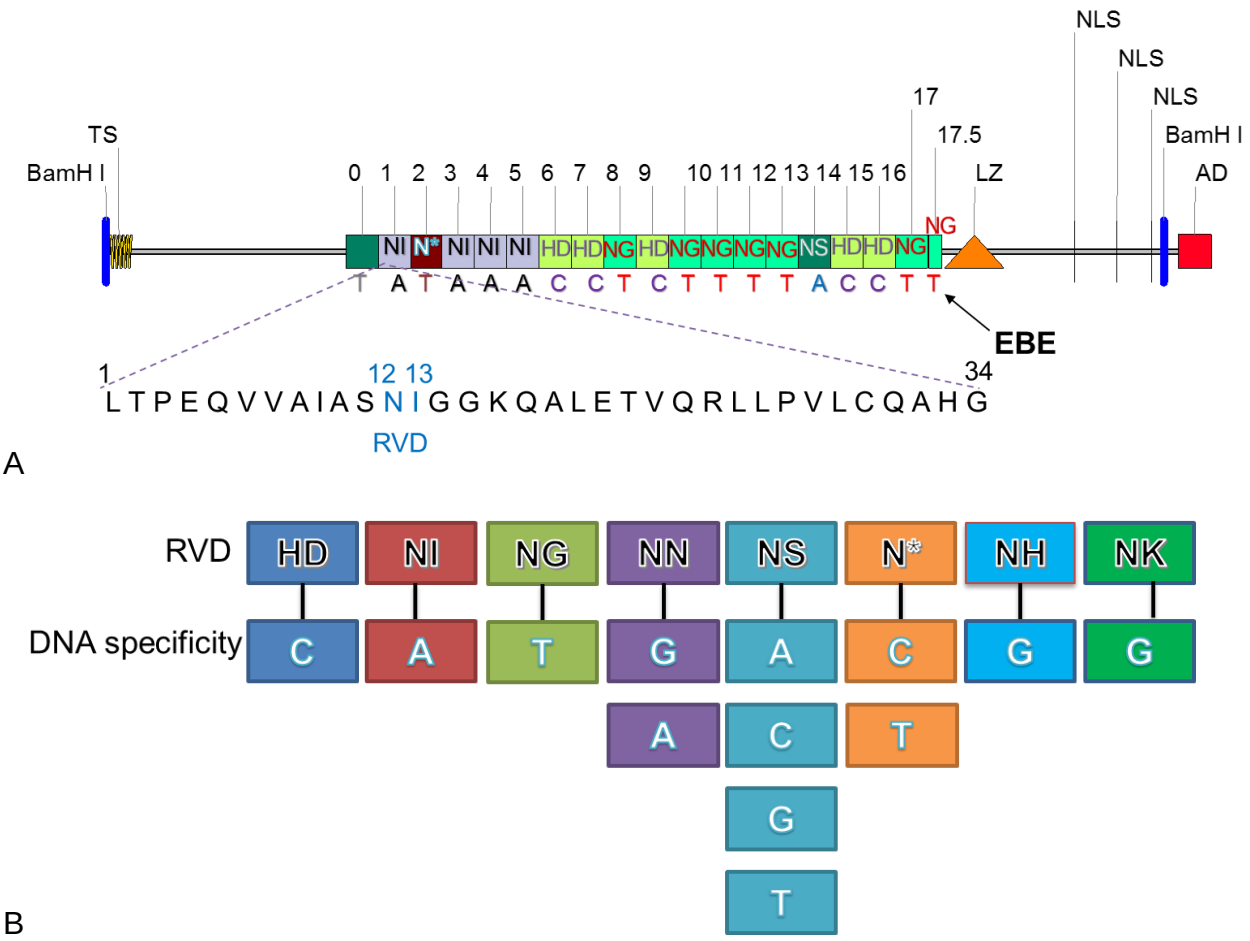


Figure 4-1. Structure features of a typical TAL effector and the DNA binding specificity. A) The functional domains that constitute TALEs and the predicted EBE exemplified by PthA4. The repeat domain is preceded by a 0th repeat, and a representative repeat containing 34 amino acid was displayed. The RVDs of each repeat and their matching bases were shown in different color. TS, translocation signal; LZ, leucine zippers; NLS, nuclear localization signal; AD, activation domain; RVD, repeat-variable di-residue. B) The most frequent RVDs and their one or several DNA bases preference. N* means missing of 13th amino acid. The RVDs and DNA bases are given as single letter abbreviation. Amino acids: H-histidine, D-aspartic acid/aspartate, N-asparagine, I-isoleucine, G-glycin, S-serine, K-lysine. Bases: C-cytosine, A-adenine, T-thymine, G-guanine.

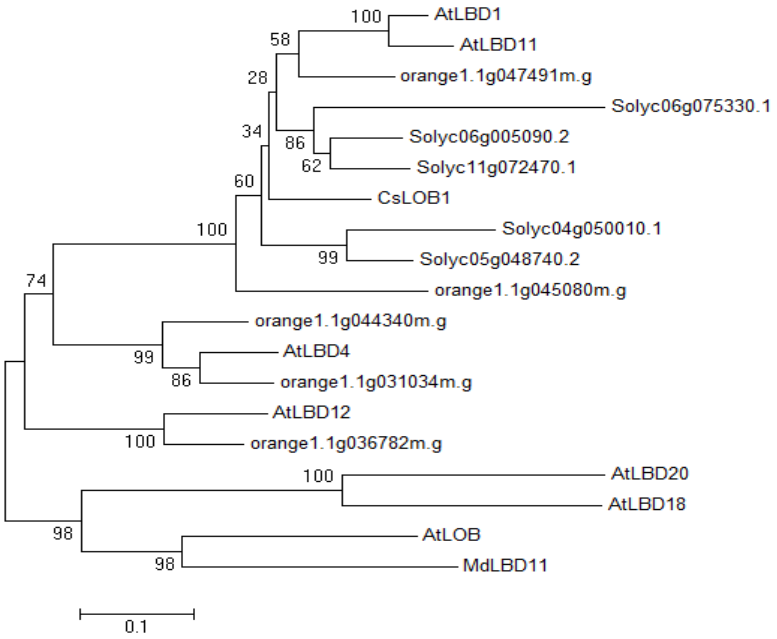


Figure 4-2. Phylogeny of LOB domain (LBD) family from *Citrus sinensis*, *Solanum lycopersicum*, and *Arabidopsis thaliana*. Five LBD family proteins from each of these three species which have highest similarity with CsLOB1 were chosen, also three LBD with identified functions was selected (AtLBD18, AtLBD20, MdLBD11). The phylogenetic tree was constructed using the neighbor-joining method with MEGA5.1 software. The numbers at the branches are bootstrap values for 1000 repeats. The scale bar represents 0.1 substitutions per sequence position.

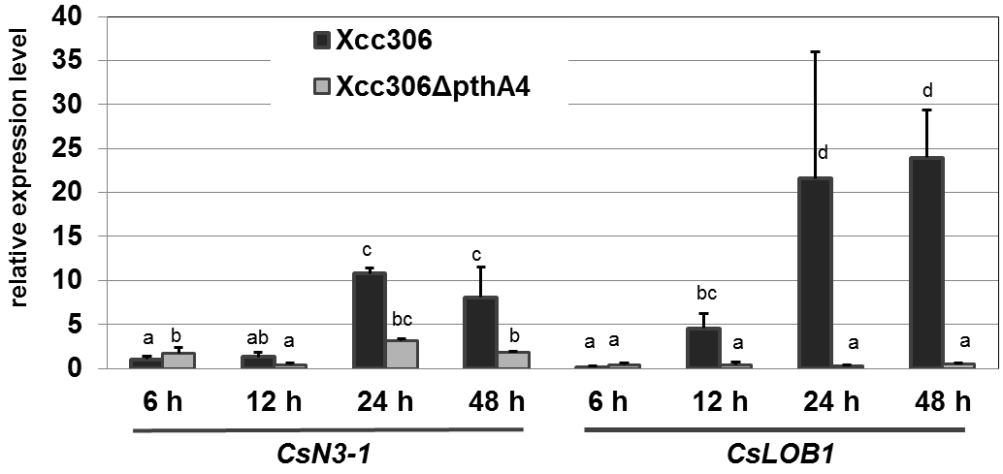


Figure 4-3. *CsN3-1* and *CsLOB1* were up-regulated in sweet orange following challenge with wild-type Xcc306 compared to Xcc306Δ*pthA4.* The expression level of *CsN3-1* and *CsLOB1* reached peak levels at 24 hours post inoculation of Xcc306. Data represent the mean ± SD; different lowercase letters represent significant differences (P≤0.01) using ANOVA analysis and Tukey test.

```
1.  CsN3Pwt       …TTCCCTATATAAACCGCTTTTGCTTCTA…ATG…
2.  CsN3PD        …TTCCCTATA    ggtacc    CTTCTA…ATG…
3.  CsN3PM1       …TTCCCTATATAAACCGCTcgaGCTTCTA…ATG…
4.  CsN3PT          // CTATATAAACCGCTTTTGCTTCTA…ATG…
5.  CsLOBPwt      …TTCTCTATATAAACCCCTTTTGCCTTAA…ATG…
6.  CsLOBPD       …TTCTCTATA    ggtacc    CCTTAA…ATG…
7.  CsLOBPM1      …TTCTCTATATAAACCCCTcgaGCCTTAA…ATG…
8.  CsLOBPT         // CTATATAAACCCCTTTTGCCTTAA…ATG…
9.  CsLOBPM2        // CTATATAAACCtCTTTTGCCTTAA…ATG…
10. CsLOBPM3        // CTATATAAACCggTTTTGCCTTAA…ATG…
11. CsLOBPins       // CTATATAAACCCCTTgTTGCCTTAA…ATG…
12. CsLOBPM5      …TTCTCgAggTAAACCCCTTTTGCCTTAA…ATG…
```

Figure 4-4. Promoter constructs used in the GUS transient expression assay. The predicted EBEs are underlined, 'Pwt' means wild-type promoter, 'PD' means deletion in promoters, 'PM' means mutation, 'PT' means truncation, and '//' represent truncated. The base mutations are in lowercase letter. The fragments were fused to the ATG of the *uidA* coding sequence.

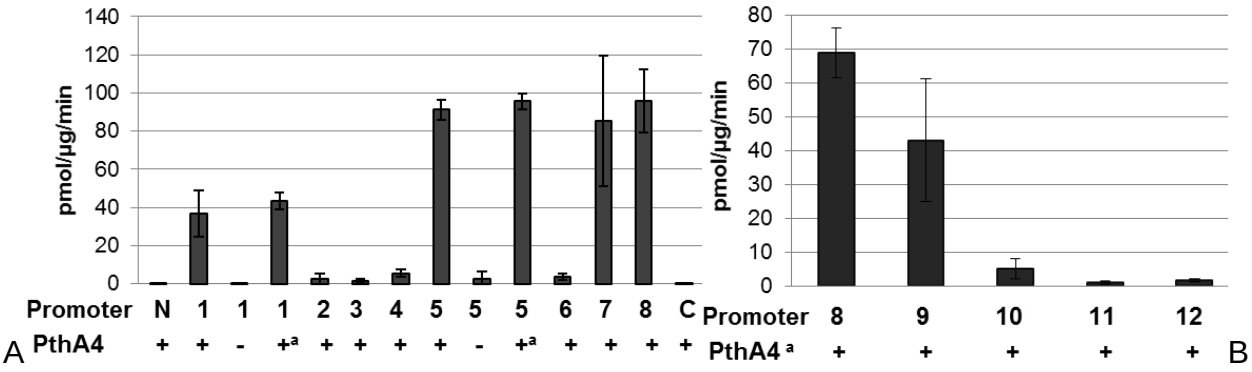


Figure 4-5. PthA4 Drives *CsN3-1* and *CsLOB1* promoter expression of *uidA* reporter gene. GUS array was measured for the inducibility of *CsN3-1* and *CsLOB1* promoter by Xcc306 and its derivative strains in sweet orange. A) The co-infiltration of Xcc306 or its derivative strains and *A. tumefaciens* carrying *CsN3-1*, *CsLOB1* promoter constructs fused with *gus* from Figure 4-4. B) Co-inoculation of Xcc306Δ*pthA4:pthA4* with the promoter constructs 8-12 in Figure 4-4. *Xanthomonas* were inoculated 5 hours after the delivery of *A. tumefaciens* containing the GUS reporter constructs as indicated in Figure 4-4. 'N' means empty vector without promoter fragment. '+' and '-' indicate with PthA4 and without PthA4 respectively, [a] represents Xcc306Δ*pthA4:pthA4*. Column C indicates the promoter of another PthA4 up-regulated probe Cit.7877.1.S1_at fused with *uidA* gene. The GUS activity was tested 5 days after inoculation. SD values are three technical replicates of one experiment, repeated twice with similar results.

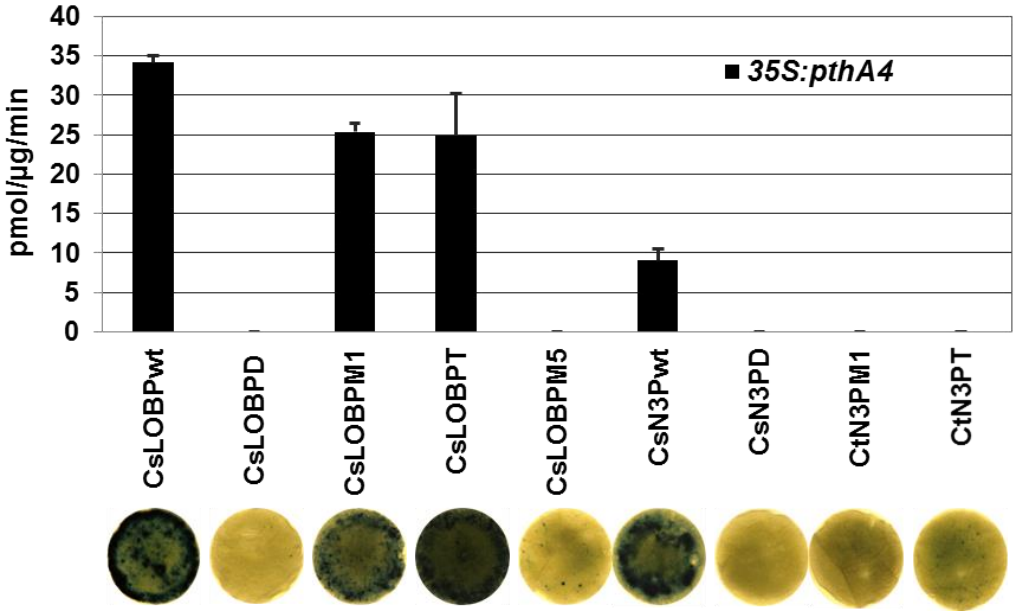


Figure 4-6. PthA4 induced the *CsLOB1* and *CsN3-1* promoter in *Nicotiana benthamiana*. The *pthA4* gene coding sequence was put downstream of CaMv35S promoter in binary vector and transformed into *A. tumefaciens*, then was co-delivered with GUS reporter constructs (see Figure 4-4) into *N. benthamiana*. The GUS assay was conducted 3 days after the inoculation. Leaf discs were stained with X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide). Error bars indicate SD.

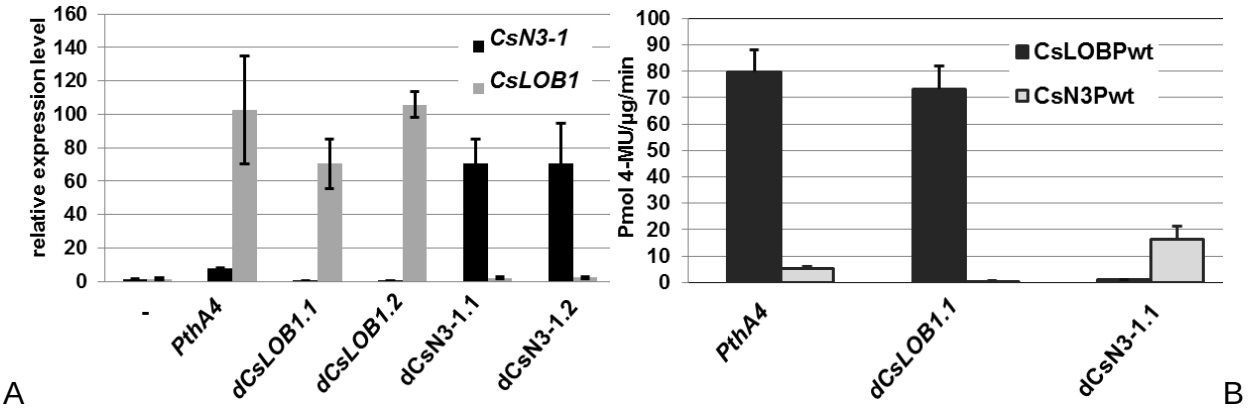
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
dCsN3-1.1 RVDs	NI	NG	NI	NI	NI	HD	HD	NN	HD	NG	NG	NG	NG	NG	NN	HD	NG	NG	HD	NG	NI	NN	NG	HD
EBE	T	A	T	A	A	A	C	C	G	C	T	T	T	T	G	C	T	T	C	T	A	G	T	C

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
dCsN3-1.2 RVDs	NG	NG	NN	NI	NI	NG	NG	HD	HD	HD	NG	NI	NG	NI	NG	NI	NI	
EBE	T	T	T	G	A	A	T	T	C	C	C	T	A	T	A	T	A	A

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
dCsLOB1.1 RVDs	NI	NI	NI	NN	HD	NI	NN	HD	NG	HD	HD	NG	HD	HD	NG	HD	
EBE	T	A	A	A	G	C	A	G	C	T	C	C	T	C	C	T	C

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
dCsLOB1.2 RVDs	NI	NG	NI	NI	NI	HD	HD	HD	HD	NG	NG	NG	NG	NN	HD	HD	NG	NG	
EBE	T	A	T	A	A	A	C	C	C	C	T	T	T	T	G	C	C	T	T

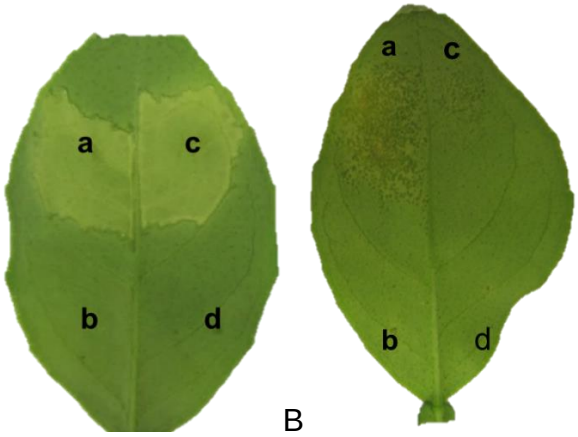
Figure 4-7. The RVDs of artificial designed TALEs (dTALes) and their targeting EBE sequences. The dTALes targeting either gene have two versions. dCsN3-1.1 targets the same position as predicted EBE_{PthA4} in *CsN3-1* promoter but with longer sequence, while dCsN3-1.2 targets at 13 bp upstream of predicted EBE_{PthA4}. The sequences targeted by dCsLOB1.1 are located 33 bp downstream of predicted EBE_{PthA4} in *CsLOB1* promoter while dCsLOB1.2 is the optimized dTALe for EBE_{PthA4} in *CsLOB1* promoter (exact match).



A B

Figure 4-8. Examination of the correctness of dTALes in qRT-PCR and GUS assay. A) Artificial dTALes induce the expression of their corresponding genes. The dTALes shown in Figure 3-7 were delivered into Xcc306Δ*pthA4* and qRT-PCR was conducted 48 h after the inoculation. B) Quantitative GUS assay using *dCsN3-1.1* and *dCsLOB1.1* complementing Xcc306Δ*pthA4* strains. The *Agrobacterium* and *Xanthomonas* were co-infiltrated into sweet orange, and the assay was conducted at 5 days after the infiltrations. Black column indicates *A. tumefaciens* with CsLOBPwt: *uidA* construct, gray column indicates *A. tumefaciens* with CsN3Pwt: *uidA* construct. The PthA4 complementing strain was used as positive control. Data represent the mean ± SD with three replicates.

TAL effector	Pustule
-	-
PthA4	+
dCsLOB1.1	+
dCsLOB1.2	+
dCsN3-1.1	-
dCsN3-1.2	-



A B C

Figure 4-9. Phenotype of dTALes complemented Xcc306Δ*pthA4* inoculation in sweet orange. A) Description of the pustule symptom mediated by several TAL effector. '-' represent Xcc306Δ*pthA4* without PthA4, and the other TAL effectors were delivered into Xcc306Δ*pthA4*. In pustule column, '-' means no visible pustule, while '+' represents pustule formation. B, C) The symptoms after infiltration of dTALes complemented strains. a, PthA4; b, Xcc306Δ*pthA4*; c, dCsLOB1.1; d, dCsN3-1.1. The strains were inoculated at the concentration of 5×10^8 (B) or 5×10^5 (C) and the leaf was photographed at 5 days or 10 days after the infiltration. The pictures were not shown for dCsLOB1.2 and dCsN3-1.2.

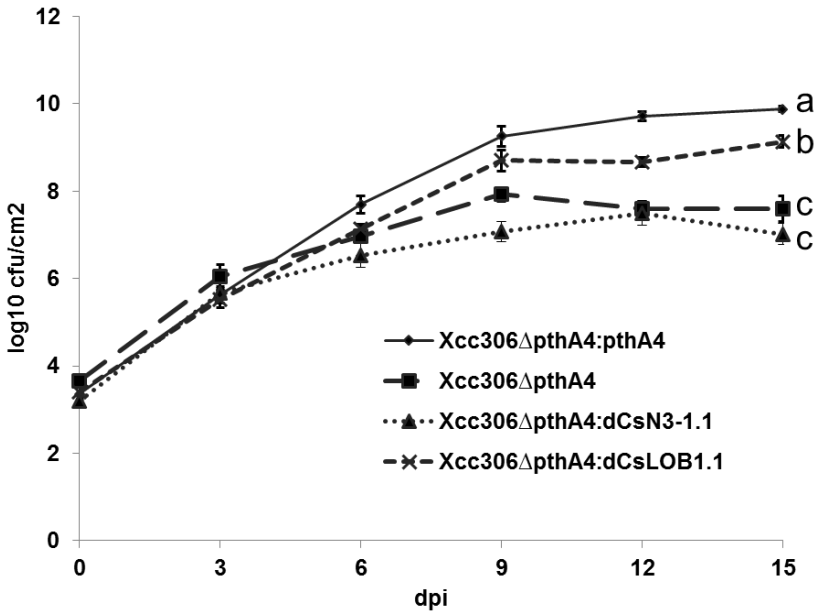


Figure 4-10. *In planta* growth of Xcc306Δ*pthA4* mutant (square) and the corresponding complemented strains. Xcc306Δ*pthA4* and Xcc306Δ*pthA4:dCsN3-1.1* showed significantly poorer bacterial growth when compared with Xcc306Δ*pthA4*:*pthA4* and Xcc306Δ*pthA4:dCsLOB1.1,* while the bacterial population of Xcc306Δ*pthA4:dCsLOB1.1* was lower than that of Xcc306Δ*pthA4*:*pthA4.* Sweet orange leaves were inoculated at the concentration of 5×10^5 cfu /ml, the population was monitored at the time points indicated. Error bars represent standard deviations (SD). The experiment was repeated twice with similar results. Significance between strains was assessed at final time point at a *P* value <0.01 by using Tukey-Kramer HSD test for post-ANOVA analysis.

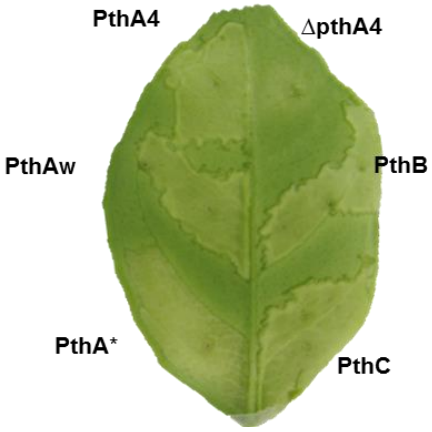


Figure 4-11. PthA4 and its homologs are critical for pustule formation on sweet orange. The *pthA4* deletion mutant Xcc306Δ*pthA4* was complemented with TAL effectors PthA4, PthAw, PthA*, PthB and PthC respectively, the symptoms cause by these complementing strains were showed. The inoculation concentration was 5×10^8 cfu/ml.

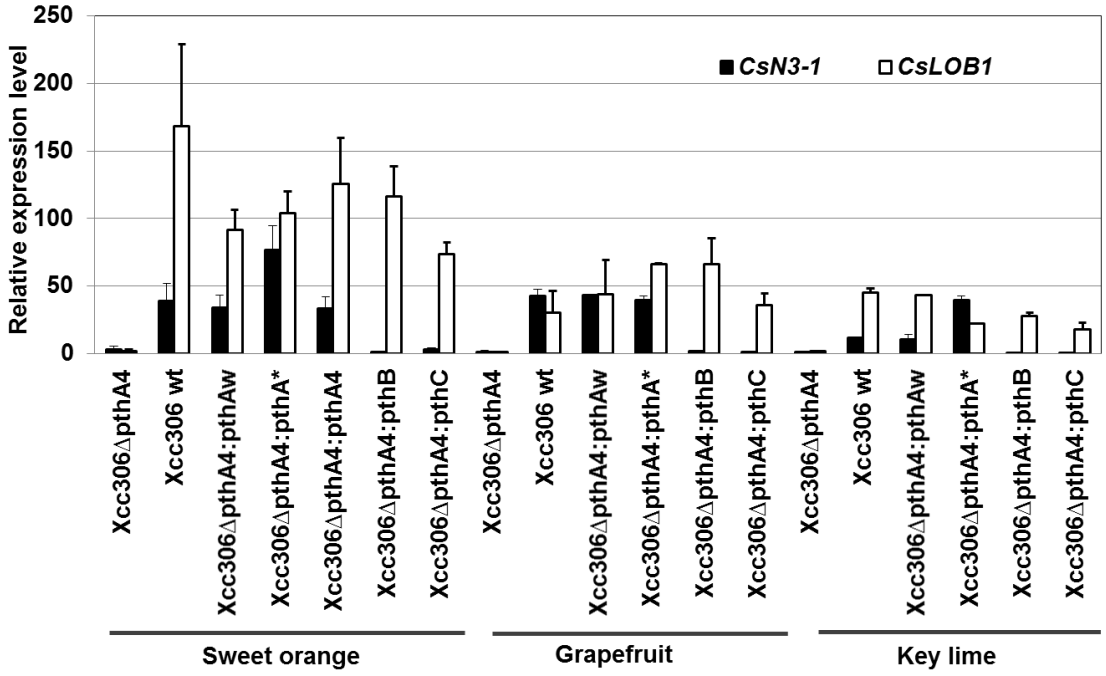


Figure 4-12. The relative expression of *CsN3-1* and *CsLOB1* induced by several TAL effectors differed in different citrus species. Black column indicates the expression of *CsN3-1* gene, and white column indicates expression of *CsLOB1* gene. RNA was prepared 48 hours post inoculation. The expression level of *CsLOB1* in grapefruit and key lime was lower than that in sweet orange after inoculation, PthB and PthC did not induce *CsN3-1* in all three species. Data represent the mean ± SD with three replications.

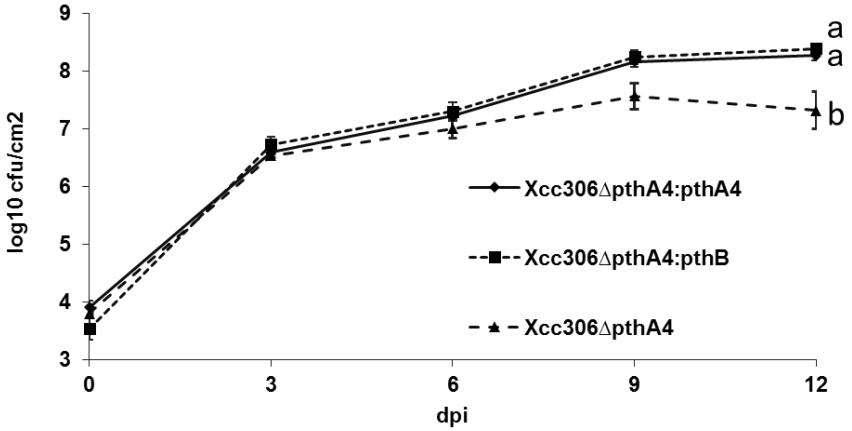


Figure 4-13. Bacterial dynamic of Xcc306Δ*pthA4* derivative strains *in planta*. The strains of Xcc306Δ*pthA4*:*pthB* and Xcc306Δ*pthA4*:*pthA4* have higher population than the mutant after 9 dpi. Sweet orange leaves were inoculated at the concentration of 5×10^5 cfu/ml. Error bars represent standard deviations (SD). The experiment was repeated twice with similar results. Different lowercase letters (a or b) mean significant differences (P≤0.01) using ANOVA analysis and Tukey test.

A

CsLOBP	T	C	T	C	T	A	T	A	T	A	A	A	C	C	C	C	T	T	T	T	G	C	C	T	T
CsN3P	T	C	C	C	T	A	T	A	T	A	A	A	C	C	G	C	T	T	T	T	G	C	T	T	C
PthA4							T	A	T	A	A	A	C	C	**T**	C	T	T	T	T	**A**	C	**C**	T	T
PthAw							T	A	T	**T**	**T**	A	C	C	**A**	C	T	**C**	T	T	**A**	C	**C**	T	T
PthA*							T	A	T	A	**C**	A	C	C	**T**	C	T	T	T	**A**	**C**	A	**T**	T	T
PthC	T	C	**T**	C	**C**	A	T	A	T	A	A	**C**	**T**	C	**C**	C	T	T	T						
PthB	T	C	**T**	C	T	A	T	**C**	T	**C**	A	A	C	C	**C**	C	T	T	T						

B

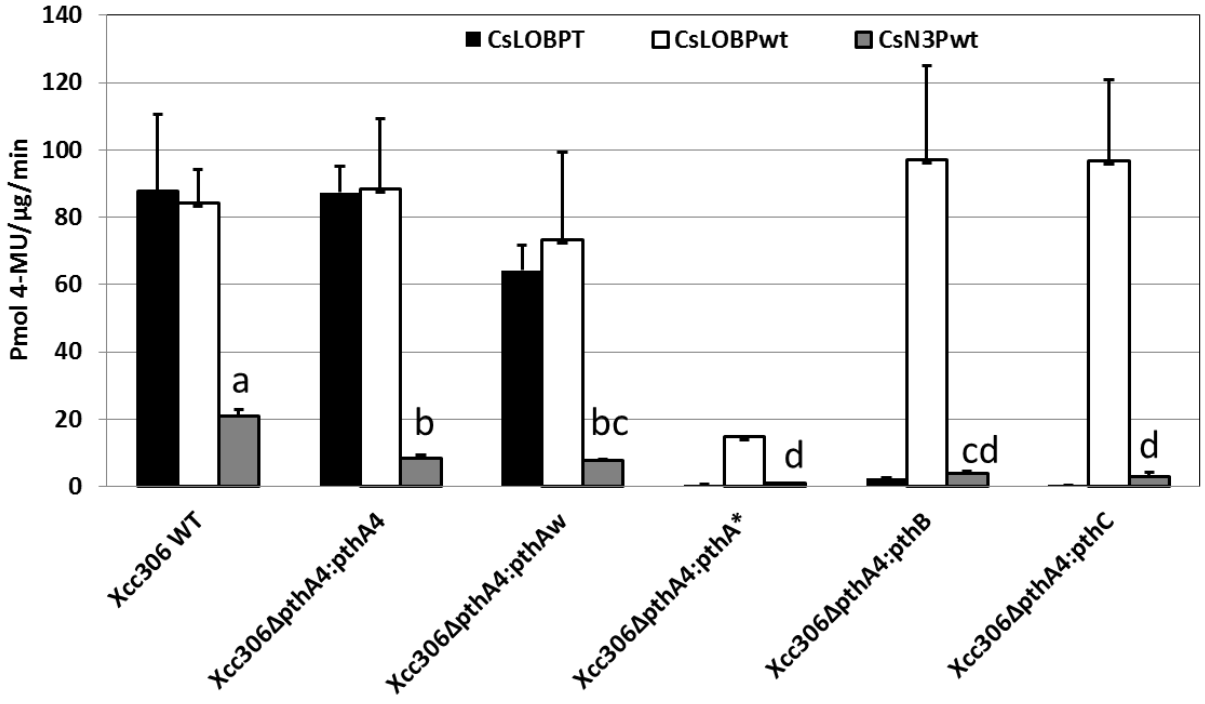


Figure 4-14. EBE of PthB and PthC in *CsLOB1* promoter is located six bases upstream of EBE_{PthA4}. A) Comparison of predicted optimized EBEs of PthA4, PthAw, PthA*, PthB, PthC (based on the TALE code) and the corresponding nucleotide sequences in *CsLOB1* and *CsN3-1* promoters. Mismatches between predicted EBE and the *CsN3-1* promoter are indicated in bold font, mismatches between predicted EBE and the *CsLOB1* promoter are indicated in gray highlight. B) GUS transient assay on sweet orange with the co-inoculation of Xcc306 or its complemented strains and *A. tumefaciens* harboring GUS constructs listing in Figure 4-4. The black, white and gray columns represent *A. tumefaciens* carrying CsLOBPT, CsLOBPwt, CsN3Pwt GUS reporter constructs respectively. Data represent the mean ± SD with three technical replicates of one experiment, repeated twice with similar results. Columns with the same lowercase letters do not differ from each other at the significance level of $P < 0.05$ using the Tukey test.

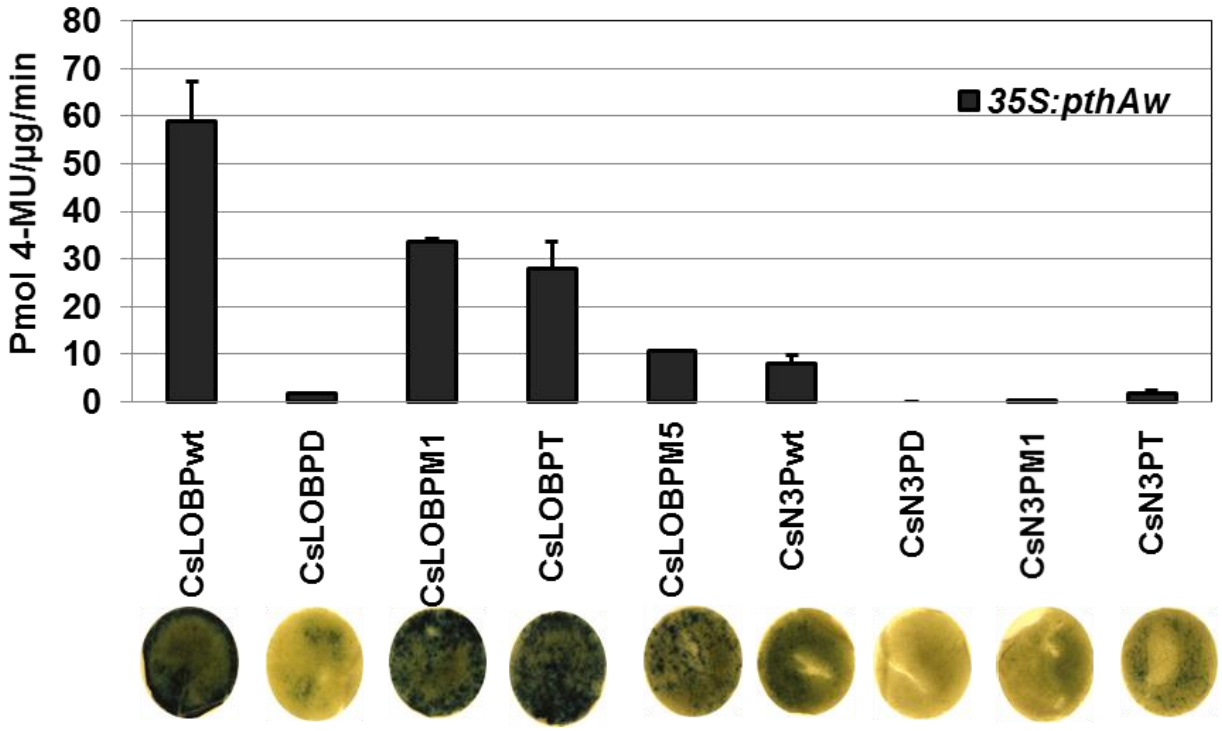
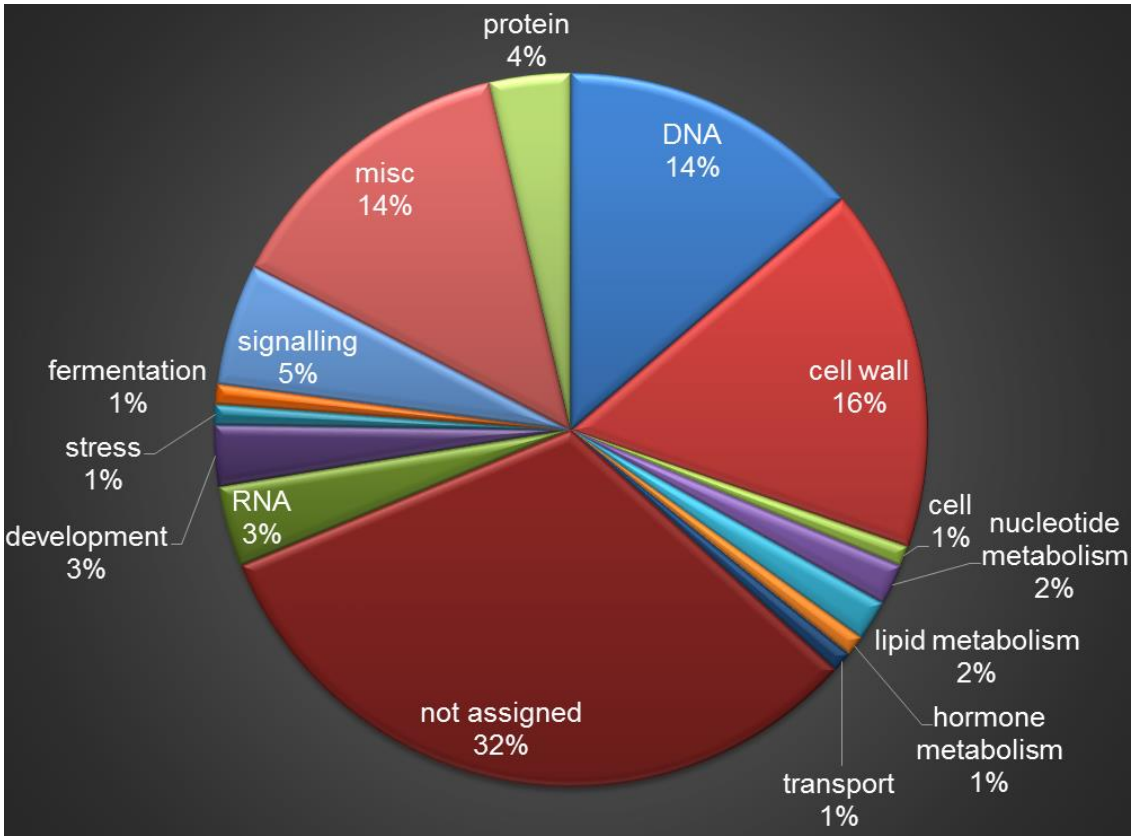


Figure 4-15. PthAw activated the *CsLOB1* and *CsN3-1* promoter in *Nicotiana benthamiana*. The *pthAw* gene coding sequence was put after CaMv35S promoter in binary vector and transformed into *A. tumefaciens*, then was co-delivered with GUS reporter constructs (see Figure 4-4) into *N*. *benthamiana*. The GUS assay was conducted 3 days after the inoculation. Leaf discs were stained with X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide). Error bars indicate SD.

A



B

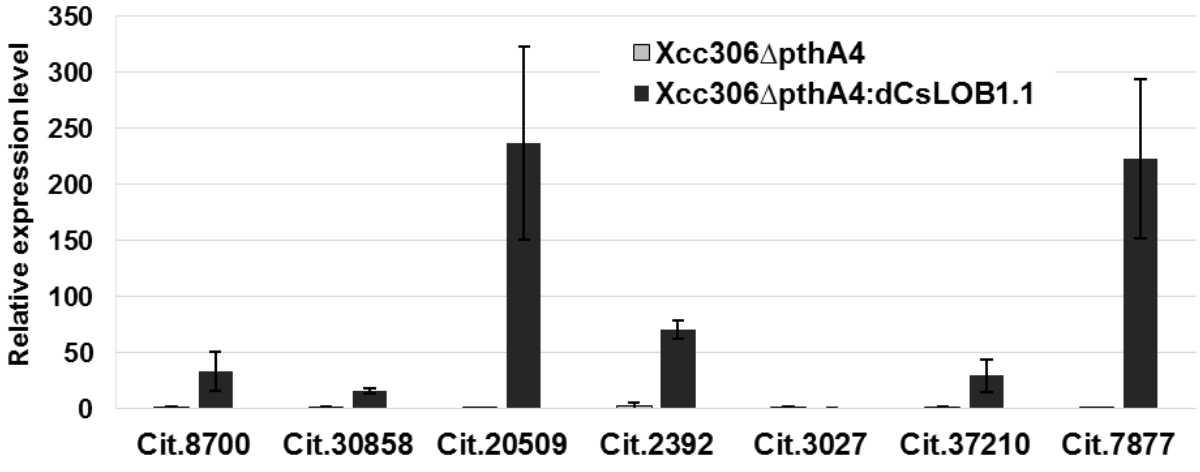


Figure 4-16. *CsLOB1* is considered to be associated with cell wall metabolism. A) Functional categories assignment of the genes induced in sweet orange by PthA4 using MapMan software. Mercator analysis was performed using the genes in sweet orange with expression fold change more than 16 in Xcc306 vs. Xcc306Δ*pthA4* (108 elements). B) Cell wall metabolism genes in sweet orange were activated by Xcc306Δ*pthA4:dCsLOB1.1* when compared with Xcc306Δ*pthA4.* The cell wall genes were selected from Table 4-2, only part of ID names were shown in label. qRT-PCR was conducted with gene specific primers at 48 h after the infiltration.

CHAPTER 5
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Citrus bacterial canker caused by *X. citri* is a devastating disease on citrus and is endemic in Florida as well as elsewhere worldwide. It is a major threat to the high valued citrus industry. Although some factors have been described, the mechanism of how the bacterial pathogen attacks the citrus host and promotes disease development is still elusive. In this study, through mutagenesis analyses, we identified PthA4 from Xcc306 acting as a major pathogenicity determinant to citrus canker, which belongs to the intensively studied TAL effector family.

The molecular responses of the citrus host plant to PthA4-mediated infection were investigated by microarray-based comparison of wild-type and *pthA4* defective strain inoculations. Genes in the categories associated with cell wall degradation, modification or disassembly, cell cycle and division, auxin responsive, protein synthesis and DNA synthesis or packaging were significantly up-regulated, while genes in the categories of photosynthesis, cytochrome P450, PR-proteins, UDP-glycosyl transferases, phenylpropanoids metabolism, ET responsive, receptor kinases were remarkably down-regulated by PthA4-dependent invasion. Among them, the induction of cell wall metabolism and cell division genes was to help the bacterial multiplication, but the activation of DNA synthesis genes was not common in other genome-wide transcriptional studies after pathogen infection. Furthermore, most of the categories that contained considerable down-regulated genes were plant defense associated, which indicates that PthA4 is associated with repression of plant defense pathways.

The targets of PthA4 were identified by analyzing the upstream region of the most highly PthA4-induced genes by using EBE_{PthA4} sequence predicted from

deciphered TALE code as the bait. Two candidate genes were captured; one gene termed as *CsLOB1* belongs to lateral organ boundaries (LOB) domain family; and another one designated as *CsN3-1* is a member of MtN3 family, which was previously reported to contain TAL effector targets. Through qRT-PCR expression experiments and gus reporter gene fusion assay, *CsLOB1* or its promoter was strongly induced by PthA4, and *CsN3-1* or its promoters was weakly activated by PthA4. In further GUS assays, the base mutation in the TATAA box and proximal 5' terminal or displacement by insertion in the binding site of *CsLOB1* promoter completely abolished induction, while 3 bases substituted at 3' terminal did not affect the activity, and the EBE upstream sequence truncation also did not affect the induction activity. These results indicate that the EBE in *CsLOB1* promoter was crucial and sufficient for the gene activation. Moreover, all the mutations in the predicted EBE_{PthA4} or truncation to the promoter of *CsN3-1* resulted in loss of induction by PthA4. Interestingly, alternate TAL effectors in other strains that determine the cause of citrus canker also targeted and activated the *CsLOB1* promoter; PthAw and PthA* seemed to share the same EBE with PthA4, while PthB and PthC targeted sequence was located 6 bp upstream. However, PthB and PthC did not drive the *CsN3-1* promoter expression. In collateral experiments, the artificial synthesized TAL effectors (dTALes) that specifically and optimally target *CsLOB1* promoter successfully activated *CsLOB1* gene and restored the pustule formation was lost by *pthA4* deletion mutant, while the dTALes targeting *CsN3-1* gene did not complement the symptoms, although they could induce the *CsN3-1* gene expression. Collectively, we consider *CsLOB1* rather than *CsN3-1* as a novo susceptibility gene that is targeted by various TAL effectors. And this is the first time to

demonstrate the member of LOB domain family as TAL effector target and susceptibility gene, while we also provide a notable example for one gene being targeted by several TAL effectors. Furthermore, in addition to the characterized functions of TAL effector target genes, which included sugar transport (*SWEET* gene) and cell enlargement (*upa20 bHLH* gene), a novel plant susceptibility pathway was explored. As the draft genomic sequences for several citrus species became available (Gmitter et al., 2012; Ollitrault et al., 2012; Xu et al., 2012), the discovery of more potential TAL effector target genes and elements downstream of *CsLOB1* will become easier.

Until now, the outstanding targets especially the EBEs of TAL effector have been employed as effective tools to control the disease. By combining the natural EBEs of multiple TAL effectors from three distinct *R* genes or adding artificial EBEs of corresponding TAL effectors into one complex *R* gene promoter, the engineered *R* gene was induced by these effectors and conferred broader spectrum disease resistance (Römer et al., 2009a; Hummel et al., 2012). But notably, it is likely that the EBEs coincidently overlap with endogenous *cis* regulatory elements, so the adding may cause unintended activation of *R* gene and result in plant aborted development. Although no major *R* genes yet described in citrus, the well characterized *avr* gene *avrGf1* suggested the presence of potential *R* gene in grapefruit and sweet orange (Rybak et al., 2009). Alternatively, we may engineer the EBEs identified from *CsLOB1* to drive the expression of *avrGf1* and transform into grapefruit or sweet orange, the *AvrGf1* will be ectopically activated in plant when encounters most of the canker-causing xanthomonads; the encoded AvrGf1 protein can be recognized by the potential R protein and trigger HR. In addition, after the identification of *S* gene, we can silence this

gene in plant to permanently suppress its expression, thus conferring reduced susceptibility to the strains containing the corresponding TAL effectors. However, we need to guarantee that silenced genes do not have important housekeeping functions in plant. The feasibility of this conception was already effectively applied in two separate studies (Yang et al., 2006a; Antony et al., 2010). In a recent report, Li et al. (Li et al., 2012) mutated the EBEs of *Os11N3* in rice by TALEN-based cleavage and gained transgenic rice lines conferring resistance to *Xoo* that contained TAL effectors AvrXa7 and PthXo3. This provides a compelling approach to produce disease resistant plants by modifying TAL effector targeted *S* genes. Furthermore, *CsLOB1* is definitely a good candidate for this approach since it is targeted by several TAL effectors, with only one single mutation being required to obtain broad spectrum resistance plant to control citrus canker; however, one of the limitations is that it may not be the only target of those TAL effectors. We also need to caution about the adverse effects in normal functions by the disruption of promoter. Last but not least, it is possible to create a gene silencing construct that is driven by TAL effector targeted promoter; when the strains containing the corresponding TAL effector attack, the targeted *S* gene will be silenced, but the same problem may happen to inactivation of its other functions, so affects plant normal growth (Doyle et al., 2013).

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BIOGRAPHICAL SKETCH

Yang Hu was born in Xinyu, Jiangxi province, China in 1986. He obtained his bachelor's degree in major plant protection from China Agricultural University in 2006. Then he got scholarship to continue his study specialized in plant resistance genes to rice blast pathogen *Magnaporthe grisea* in Department of Plant Pathology from the same university, and he acquired Master of Science in 2009. After that, in the same year, Hu was awarded research assistantship to pursue his doctoral studies in Department of Plant Pathology in University of Florida. During the Ph.D. period, he worked with Dr. Jeffrey B. Jones and focused on the mechanism of *Xanthomonas* virulence in causing citrus canker.