FUNCTIONAL ANALYSIS OF BACTERIAL TAL EFFECTORS AND THE TARGETED SUSCEPTIBILITY GENES IN PLANTS

by

JUNLI ZHANG

B.S., Hebei University, China, 2006
M.S., Institute of Microbiology, Chinese Academy of Sciences, China, 2009

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2015
Abstract

The genus *Xanthomonas* consists of bacterial species causing economically important plant diseases in major crops. In a wide variety of *Xanthomonas* species, the transcription activator-like (TAL) effectors (proteins) are synthesized and secreted into host cells, whereby they enter the plant nucleus. TAL effectors bind specific host gene promoters, inducing the expression of the targeted genes, which in some cases leads to either resistance or an enhanced state of disease susceptibility. The TAL effectors in individual *Xanthomonas* species and their targets in host plants have been characterized in relatively few cases. The premier example is the induction of any one member of a clade of sugar transporter genes in rice by TAL effectors of the bacterial blight pathogen *X. oryzae* pv. *oryzae*, where induction of the susceptibility (S) genes was shown to be required for the disease process. TAL effector genes are present in a wide variety of *Xanthomonas* species other than *X. oryzae* pv. *oryzae*. My dissertation focuses on the characterization of the TAL effectors in the citrus bacterial canker (CBC) and soybean bacterial pustule pathosystems. In CBC, *CsLOB1* was identified as the S gene targeted by multiple major TAL effectors from CBC causal strains. Furthermore, another two members in family of citrus LBD family, although not identified as targets in the field, can serve as S genes in CBC. Initial analysis of bacterial pustule disease of soybean indicates that the TAL effector TAL2 of *X. axonopodis* pv. *glycines* is a virulence effector and associated with the expression of two candidate S genes, which encode a member of the ZF-HD transcription factors and a member of aluminum activated malate transporter family. These studies will enhance our understanding of plant-bacterial interactions and evolution of disease susceptibility, and also inform development of durable disease resistant crop varieties.
FUNCTIONAL ANALYSIS OF BACTERIAL TAL EFFECTORS AND THE TARGETED SUSCEPTIBILITY GENES IN PLANTS

by

JUNLI ZHANG

B.S., Hebei University, China, 2006
M.S., Institute of Microbiology, Chinese Academy of Sciences, China, 2009

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2015

Approved by:
Major Professor
Frank White
Abstract

The genus *Xanthomonas* consists of bacterial species causing economically important plant diseases in major crops. In a wide variety of *Xanthomonas* species, the transcription activator-like (TAL) effectors (proteins) are synthesized and secreted into host cells, whereby they enter the plant nucleus. TAL effectors bind specific host gene promoters, inducing the expression of the targeted genes, which in some cases leads to either resistance or an enhanced state of disease susceptibility. The TAL effectors in individual *Xanthomonas* species and their targets in host plants have been characterized in relatively few cases. The premier example is the induction of any one member of a clade of sugar transporter genes in rice by TAL effectors of the bacterial blight pathogen *X. oryzae pv. oryzae*, where induction of the susceptibility (S) genes was shown to be required for the disease process. TAL effector genes are present in a wide variety of *Xanthomonas* species other than *X. oryzae pv. oryzae*. My dissertation focuses on the characterization of the TAL effectors in the citrus bacterial canker (CBC) and soybean bacterial pustule pathosystems. In CBC, *CsLOB1* was identified as the S gene targeted by multiple major TAL effectors from CBC causal strains. Furthermore, another two members in family of citrus LBD family, although not identified as targets in the field, can serve as S genes in CBC. Initial analysis of bacterial pustule disease of soybean indicates that the TAL effector TAL2 of *X. axonopodis pv. glycines* is a virulence effector and associated with the expression of two candidate S genes, which encode a member of the ZF-HD transcription factors and a member of aluminum activated malate transporter family. These studies will enhance our understanding of plant-bacterial interactions and evolution of disease susceptibility, and also inform development of durable disease resistant crop varieties.
# Table of Contents

Table of Contents........................................................................................................................................v

List of Figures..................................................................................................................................................ix

List of Tables..................................................................................................................................................xi

Acknowledgments............................................................................................................................................xii

Chapter 1 - TAL effectors and their host targets.......................................................................................1

Abstract.......................................................................................................................................................1

Introduction..................................................................................................................................................1

TAL effectors and S genes.................................................................................................................................4

S genes in rice bacterial blight disease.........................................................................................................4

S genes in cassava bacterial blight disease..................................................................................................6

S genes in rice bacterial leaf streak disease...............................................................................................7

S genes in bacterial spot disease of pepper.................................................................................................8

TAL effectors and E genes.................................................................................................................................9

E proteins are not homologs of classical R proteins...................................................................................10

E genes in bacterial spot disease on pepper...............................................................................................11

E genes in bacterial blight disease of rice.....................................................................................................12

Summary and discussion................................................................................................................................15

Chapter 2 - Functional analysis of TAL effector PthAw in citrus bacterial canker........................................28

Abstract.......................................................................................................................................................28

Introduction..................................................................................................................................................28

Methods and materials.................................................................................................................................32
Plant material, bacterial strains, and plasmids.......................... 32
Bacterium inoculation................................................................ 33
Designed TAL effector construction......................................... 33
RNA extraction, reverse transcription and quantitative RT-PCR analysis........ 33
Microarray assays.................................................................... 34
Candidate gene retrieval and promoter analysis.......................... 34
Thin sectioning........................................................................ 34
Bacterial population assay in citrus plants................................. 35
Results..................................................................................... 35
PthAw is a virulence effector in Xcc306.................................... 35
Identification of the candidate targeted S gene of PthAw using microarray assay..... 35
Validation of the S genes using designed TAL effectors................. 36
The promoter of S gene CsLOB1 contains EBEs for multiple TAL effectors in CBC. 37
Discussion................................................................................ 37

Chapter 3 - Homologs of CsLOB1 in citrus function as disease susceptibility genes in citrus canker......................................................... 45

Abstract...................................................................................... 45

Introduction................................................................................. 45

Methods and materials................................................................ 48
Phylogenetic analysis of C. sinensis LBD proteins........................... 48
Phylogenetic analysis of LBD proteins of CsLOB1-4 and the closely related LBD members in A. thaliana................................................................. 49
RNA-Seq analysis....................................................................... 49
Results........................................................................................................................................50

Phylogeny of citrus LBD gene family...........................................................................................50

Pustule symptoms are restored by dTALes targeting CsLOB2 and CsLOB3 but not
CsLOB4.......................................................................................................................................50

The dTALe dCsLOB2 promoted an increase in bacterial populations on citrus leaves 51
S gene expression does not contribute to enhanced virulence of a non-host pathogen. 52

Comparison of genes induced in association with CsLOB1 or CsLOB2 expression.....52

Validation of the induction of cell wall related genes in association with CsLOB1-4.. 53

Discussion...................................................................................................................................55

Chapter 4 - Functional analysis of TAL effectors in X. axonopodis pv. glycines.................74

Abstract......................................................................................................................................74

Introduction..................................................................................................................................74

Methods and materials................................................................................................................76

Plant material, bacterial strains, and plasmids...........................................................................76

Genomic DNA extraction from Xag12-2 strains.........................................................................76

TAL effector cloning.....................................................................................................................77

Xag12-2 TAL effector gene mutagenesis....................................................................................77

Plant inoculation..........................................................................................................................77

Bacteria population assays.........................................................................................................78

RNA-Seq assays..........................................................................................................................78

Candidate gene promoter retrieval and analysis.......................................................................79

Results.........................................................................................................................................79

The TAL effector profile of Xag12-2.........................................................................................79
The virulence of Xag12-2 was altered by loss of TAL2........................................79
Candidate targeted S genes of TAL2..................................................................................80
Summary and discussion........................................................................................................81
References........................................................................................................................................89

Appendix A - Strain, plasmids and primers used in the projects.........111
List of Figures

Figure 1-1  The structure of a prototypic TAL effector................................................................. 21
Figure 1-2  The code for common RVDs and the associated nucleotides................................. 21
Figure 1-3  Promoters of S genes and nucleotide polymorphisms in dominant and recessive alleles.... 23
Figure 1-4  Promoter of E genes and polymorphisms in dominant and recessive alleles............... 25
Figure 1-5  A phylogenetic tree of BS3 related proteins................................................................. 26
Figure 1-6  Structural predictions for group 2 E proteins............................................................... 27
Figure 2-1  Pustule formation induced by the Xcc306 strains carrying PthAw effector............... 40
Figure 2-2  Potential EBEs in promoters of orange1.1g025686m and orange1.1g026556m........ 40
Figure 2-3  Validation of induction of CsLOB1 and CsSWEET1 by PthAw. ............................ 41
Figure 2-4  Pustule formation is dependent on CsLOB1............................................................... 42
Figure 2-5  In planta growth of Xcc306ΔpthA4 mutant (square) and the corresponding complemented strains...................................................................................................................... 43
Figure 2-6  EBE predictions of the five TAL effectors on promoter of CsLOB1.............................. 43
Figure 3-1  Phylogenetic tree of C. sinensis LBD genes................................................................. 59
Figure 3-2  DTALe design and construction for CsLOB2, CsLOB3 and CsLOB4........................... 60
Figure 3-3  The targeted LBD genes are induced by the respective dTALes................................. 61
Figure 3-4  Disease symptoms induced by dTALes targeting the three CsLOB1-related genes. ...... 62
Figure 3-5  DCsLOB2 promoted the increase of bacterial population in host plants....................... 63
Figure 3-6  CsLOB1 is not induced by the dTALes targeting CsLOB2 or CsLOB3......................... 64
Figure 3-7  S gene expression does not contribute to enhanced virulence of a non-host pathogen. ...... 65
Figure 3-8  RNA-Seq results of gene expression profile induced by CsLOB1 and/ or CsLOB2........ 66
Figure 3-9  Mercator functional category assignments of common genes that up regulated in association with \textit{CsLOB1} and \textit{CsLOB2} expression. ................................................................. 67

Figure 3-10  Mapman metabolic overview of the genes regulated by LBD family members. .................69

Figure 3-11  QRT-PCR validation of cell wall-related genes induced by d\textit{CsLOB1}, d\textit{CsLOB2}, d\textit{CsLOB3} or d\textit{CsLOB4}...........................................................................................................................................71

Figure 3-12  LBD proteins of \textit{CsLOB1}, \textit{CsLOB2}, \textit{CsLOB3}, \textit{CsLOB4} and closely related LBD members in \textit{A. thaliana}...........................................................................................................................................73

Figure 4-1  TAL effector profile of strain Xag12-2................................................................................83

Figure 4-2  Strain virulence is reduced in a \textit{Tal2} mutant. .................................................................85

Figure 4-3  RNA expression assay of soybean leaves treated with WT or S12 with or without CHX...87

Figure 4-4  Potential EBEs in promoters of Glyma20g04880 and Glyma05g35190.............................88
List of Tables

Table 2-1 Top seven PthAw up-regulated genes................................................................. 44
Table 4-1 Top ten up-regulated genes by TAL2 from RNA-Seq data analysis results......... 86
Table A-1 Strains and plasmids used in the study............................................................... 111
Table A-2 Primers used in this study.................................................................................. 113
Acknowledgments

It is my pleasure to express my deepest gratitude to my supervisor Dr. Frank White for his inspiring guidance and persistent help on my research. He has been always available when I need help for my research and has been always giving enlightened advice to push my project forward. I also want to say thank you to my committee members Dr. Bikram Gill, Dr. Anna Whitfield, Dr. Sunghun Park and Dr. Sanzhen Liu who have been very helpful with my project research and thesis writing.

I am extremely grateful to my lab members Dr. Ken Obasa, Mr. Zhao Peng, Dr. Ginny Antony and Dr. Tariq Mahmood for their generous help and readily corporation. I also want to say thank you to the undergraduate students who used to work in the lab especially Ms. Daniela Guereca and Mr. Eric Acosta who were my research assistants during my Ph.D stage and helped me a lot with my lab experiments and saved my time for writing. I acknowledge my collaborators in University of Florida Dr. Jeffrey B. Jones, Dr. Nian Wang and Dr. Yang Hu for their selfless data sharing and material supply.

I would love to thank to the K-State Integrated Genomics Facility, the members have been so easy to reach for suggestions and help with my research. I also express my appreciation to the office staff and computer lab for their time, help and support, which has made my Ph.D much easier.

I am extremely thankful to my husband, mom, dad, sister, brother and all my friends for their endless love and support.
Chapter 1 - TAL effectors and their host targets

Abstract

Transcription activator-like (TAL) effectors are a subset of type III effector proteins that are translocated from pathogenic bacteria into plant host cells, whereby they enter into the nucleus and bind to specific regions of targeted gene promoters. TAL effectors either enhance susceptibility in hosts through activating the host susceptibility (S) genes or elicit resistance reactions by promoting expression of effector-specific host resistance genes, known as executor resistance (E) gene. Based on the features of the S gene products, two general categories have been proposed. One category includes sugar transporters of the SWEET gene family and a sulphate transporter gene in rice. The second group consists of genes encoding members of different transcription factor families, including upa20 from pepper, encoding a bHLH transcription factor, and a bZIP transcription factor gene OsTFX1 from rice. E genes have been classified into two groups based on the protein products. Group 1 consists of BS3, which is predicted to have a catalytic function. Group 2 consists of relatively short proteins with multiple hydrophobic domains and no apparent catalytic functions. S genes, E genes and TAL effector genes facilitate interplay of the selective pressures on the host and pathogen.

Introduction

Xanthomonas infects many monocotyledonous and dicotyledous plant species, and the pathogenicity, in most species, depends on the effector proteins secreted by a type III secretion (T3S) system (Leyns et al., 1984; Tampakaki et.al., 2004). An important family of Xanthomonas T3S effectors is the transcription activator-like (TAL) effector family. TAL effectors function as host gene specific transcription factors that can target both cognate susceptibility (S) and/or resistance (R) gene, leading to enhanced expression and consequential phenotypic effects (Gu et al., 2005; Yang et al., 2006; Kay et al.,
TAL effector genes are limited to members of the genus *Xanthomonas* and *Ralstonia* (De Feyter et al., 1993; Heuer et al., 2007; Hopkins et al., 1992; Salanoubat et al., 2002). The genes are ubiquitous in some species and have apparent critical functions in a number of diseases (Yang et al., 2004; Cernadas et al., 2014, Hu et al., 2014; Cohn et al., 2014; Schwartz et al., 2015).

Upon infection with the *Xanthomonas* pathogens, TAL effectors enter into the cell nuclei where they bind to the sequence specific promoter regions, here designated as effector binding elements (EBEs), of the targeted host genes and elevate the expression of the genes. Some targeted host genes, upon expression, have measurable effects on disease symptomology and/or on the ability of the bacteria to multiply on the host and are referred as disease S genes (Antony et al., 2010a; Boch et al., 2014; White et al., 2009b; Yang et al., 2006). On the other hand, some TAL effectors induce hypersensitive reaction, or HR, on the respective host plants and restrict pathogen growth at the site of infection on cultivars containing TAL effector-specific resistance genes (Gu et al., 2005; Tian et al., 2014; C. Wang et al., 2014). Resistance genes that are TAL effector dependent for expression have been designated as terminator or executor resistance (E) genes (Bogdanove et al., 2010; Tian et al., 2014). S genes or E genes and the cognate TAL effectors follow a gene-for-gene, and, in some cases, genes-for-gene model analogous to the avirulence (Avr) gene and resistance (R) gene interactions in other pathogen/plant interactions (Boch et al., 2014; White et al., 2009b).

The TAL effectors have a conserved N-terminus with an essential domain for type III effector secretion, a C-terminus with functional nuclear localization signal motifs and a potent eukaryotic acidic transcription activation domain, and central repetitive region, which controls DNA binding and TAL effector specificity. The repetitive domain consists of 33 to 35 amino acid repeats and are polymorphic at amino acid residues 12 and 13, which are referred to as the repeat-variable di-residues (RVD) (Figure 1-1). Each TAL effector repeat corresponds to one DNA base in the EBE, which is stabilized by amino
acid residue 13 and termed as the base-specifying residue (de Lange et al., 2014; Deng et al., 2012; Mak, et al., 2012). Proximal to the N-terminal portion of the repetitive domain are non-canonical repeats that mediate non-specific TAL effector–DNA interaction and pairing with the initial 5’ invariant nucleotide thymine (Boch et al., 2009; Gao et al., 2012; Moscou et al., 2009). The most common RVDs are NI, NG, HD, NN, NK, NS and N* (N represents Asparagine, I represents isoleucine, G represents Glycine, D represents aspartic acid, K represents Lysine, S represents Serine, * represents no amino acid residue at what would otherwise be position 13).

Repeats with various RVDs have preferences for different nucleotides, and a rudimentary code for RVDs and the corresponding nucleotides have been elucidated. Using the simple code, artificially designed TAL effectors (dTALes) have been developed by replacing the middle repeat parts with new DNA segments targeting specific EBEs of interest (Li et al., 2012b; Li et al., 2013; Streubel et al., 2013). Additional RVDs and the corresponding nucleotides have been reported (Figure 1-2), and public online services for searching the EBEs for the known TAL effector sequences have been developed (Grau et al., 2013). Two representative online services are the TAL Effector Nucleotide Targeter 2.0 from Cornell University (Doyle et al., 2012), and the TALgetter web-server (Grau et al., 2013). At the same time, numerous methods have been developed to construct TAL effector genes. The major principle behind several methods is to build modules of 2, 6, 8 or 10 oligomers, which are combined into longer components, and then ligated into the TAL effector gene backbone vector (Cermak et al., 2011; Li et al., 2011; Morbitzer et al., 2011; Weber et al., 2011; Zhang et al., 2011). A method without ligation was also developed (Schmid-Burgk et al., 2013). The dTALes have been used as transcription factors to either activate or repress the transcription of user-defined targeted genes (Cong et al., 2012; Geissler et al., 2011; Li et al., 2013; Streubel et al., 2013).
TAL effectors and S genes

Since their discovery, a variety of TAL effectors have been demonstrated to be either avirulence or virulence factors or both. The first TAL effector AvrBs3 was identified on the basis of the interaction with the resistance gene in pepper Bs3 (Bonas et al., 1989). The first two TAL effector genes that were known to contribute measurable phenotypic effects to the bacterial infection in the absence of resistance responses were PthA and Avrb6 (Yang et al., 1994). Subsequently, a variety of cognate targeted host genes of some TAL effectors, principally in Xanthomonas/rice diseases, were identified. Generally, known S genes can be categorized into two groups, genes in group 1 are transporter genes for sugar transporter of the SWEET family and one sulphate transporter gene. Group 2 genes encode transcription factors, including UPA20, a member of bHLH transcription factor family, and OsTFX1, a member of the bZIP transcription factor family (Kay et al., 2007; Sugio et al., 2007). Elevated expression of sugar transporter genes, originally identified as members of the nodulin 3 (N3) family from Medicago, and known now as SWEET genes, are critical for full disease symptom in bacterial blight diseases of rice and cassava (Anthony et al., 2010a; Cohn et al., 2014; Streubel et al., 2013; Yang et al., 2006; Yu et al., 2011; Zhou et al., 2015; Chen et al., 2010). Curiously, both causal pathogens, X. oryzae pv. oryzae and X. axonopodis pv. manihotis, respectively, invade the vascular tissues in their hosts. The gene OsSULTR3;6, encoding a sulphate transporter, was identified in rice bacterial leaf streak disease, which is incited by the non-vascular pathogen X. oryzae pv. oryzicola (Cernadas et al., 2014). Whether SWEET gene functions, per se, are involved in a xylem-specific function is unknown.

S genes in rice bacterial blight disease

Bacterial blight of rice is an endemic pathogen in most of the world rice growing regions. The vascular pathogen enters the plant through hydathodes and wounds, multiplies in the intercellular spaces of the underlying epithem, and enters the xylem, spreading systemically (Mew, 1987; Niño-Liu et al.,
TAL effectors that have an apparent critical function in virulence have been termed major TAL effectors. TAL effectors play a critical role in bacterial blight disease of rice (White et al., 2009b). Loss of TAL effector PthXo1 function due to mutations in the corresponding gene pthXo1 significantly reduces the virulence of the strain (Yang et al., 2004). The rice gene Os8N3/OsSWEET11 on chromosome 8 was shown to be the direct target of PthXo1, and the EBE was found 45bp upstream of the TATA box in the promoter of the gene. Strain PXO99A cannot induce the expression of Os8N3 on the near-isogenic rice line IRBB13 due to a 243-bp insertion of DNA that disrupts the PthXo1 EBE (Figure 1-3A) (Roemer et al., 2010; Yang et al., 2006; Antony et al., 2010a). Three additional major TAL effectors of X. oryzae pv. oryzae are PthXo2 from strain JXO1, AvrXa7 from Philippine strain PXO86, and PthXo3 from the Philippine strain PXO61 (Yang et al., 2004). The genes for any one of these effectors can complement a pthXo1 mutant of PXO99A for virulence by targeting alternate SWEET gene Os11N3/OsSWEET14. PthXo3 and AvrXa7 recognize overlapping EBEs at the TATA box of Os11N3/OsSWEET14 on rice chromosome 11 (Figure 1-3B) (Antony et al., 2010a; Roemer et al., 2010). Two additional major TAL effectors, TalC and Tal5, were identified in X. oryzae pv. oryzae strains of African origin that also target Os11N3/OsSWEET14, suggesting an evolutionary convergence on the same key target (Chu et al., 2006; Streubel et al., 2013; Yu et al., 2011). PthXo2 targets OsSWEET13 on chromosome 12 (Zhou et al., 2015). Further analysis using dTALes revealed two close paralogs - OsSWEET12 and OsSWEET15 - in the same clade with Os8N3/OsSWEET11, OsSWEET13 and Os11N3/OsSWEET14 could serve as potential S genes for X. oryzae pv. oryzae, while the paralogs in other clades could not (Streubel et al., 2013).

TAL effectors hijack otherwise normal host genes for the benefit of the disease process. However, the biochemical functions of the SWEET genes in the disease process are unknown. Members of clade III SWEET genes have been shown to transport sucrose preferentially (Chen et al., 2012). Sugar
transport may have a nutritional benefit to pathogenic bacteria (Chen et al., 2010). Os8N3/OsSWEET11 was also shown to be involved in copper redistribution, which might help to overcome rice defenses against X. oryzae pv. oryzae invasion (Yuan et al., 2010). The actual benefit to the invading pathogens has not been determined.

PthXo6 was demonstrated to be a common TAL effector in a wide variety of X. oryzae pv. oryzae strains and contributes virulence to X. oryzae pv. oryzae. PthXo6 specifically induces a bZIP transcription factor gene OsTFX1 in a wide range of rice lines (Sugio et al., 2007). The EBE overlaps with the TATA box (Figure 1-3C) (Roemer et al., 2010). Ectopic expression of OsTFX1 in rice plants conferred susceptibility to the host plants in the absence of PthXo6 (Sugio et al., 2007). Related homologs in other species indicate that bZIP transcription factors might function in regulating developmental and physiological response pathways (Siberil et al., 2001). The role played by OsTFX1 in infection or normal plant physiology is unknown.

**S genes in cassava bacterial blight disease**

*X. axonopodis* pv. *manihotis* is a foliar and vascular pathogen that penetrates the leaf tissue through stomata or wounds, and enters the xylem, causing shoot wilt and leaf blight symptoms (Lozano et al., 1974; Verdier et al., 2004). Most *X. axonopodis* pv. *manihotis* strains contain one to five TAL effector genes (Bart et al., 2012; Castiblanco et al., 2013). Three TAL effector genes were reported to contribute to virulence to *X. axonopodis* pv. *manihotis* strains, including TALE1\textsubscript*Xam*, TAL2\textsubscript*Xam668* and TAL14\textsubscript*Xam668*. The gene HsfB3, encoding a heat shock transcription factor, is the gene with the highest gene induction level in the presence of TALE1\textsubscript*Xam*. TAL14\textsubscript*Xam668* was proposed to target gene 007568a, encoding a pectate lyase, and was chosen based on the expression profile. However, the functional requirements of HsfB3 and 007568a in host susceptibility remain to be determined (Bart et al., 2012; Castiblanco et al., 2013).
TAL20_{Xam668} was shown to influence both bacterial growth and symptom development (Cohn et al., 2014). A RNA-Seq screen revealed that the host gene MeSWEET10a was a potentially targeted gene for TAL20_{Xam668}. The candidate EBE, which overlaps the TATA box, was tested using Agrobacteria-mediated transient expression and electro-mobility shift assays (Figure 1-3D). The role of MeSWEET10a as an S gene was functionally validated using dTALes targeting two other EBEs in the promoter of MeSWEET10a. A phylogenetic analysis placed MeSWEET10a in the same clade as the clade III S genes of bacterial blight of rice. MeSWEET10a is the only S gene in the SWEET family outside of rice, indicating possible relationship between the disease processes incited by X. oryzae pv. oryzae and X. axonopodis pv. manihotis (Cohn et al., 2014).

**S genes in rice bacterial leaf streak disease**

*X. oryzae* pv. *oryzicola* strains enter host plants mainly through stomata or wounds, multiply in the sub-stomatal space, and colonize the mesophyll apoplast. *X. oryzae* pv. *oryzicola*, therefore, differs from *X. oryzae* pv. *oryzae* in that it does not spread in the xylem. The typical symptom is water-soaked lesions along the leaf between the veins, which then coalesced into necrotic streaks (Niño-Liu et al., 2006). Various *X. oryzae* pv. *oryzicola* strains carry large repertoires of TAL effector genes, ranging in number from 25 to 30 (Gonzalez et al., 2007). The sequenced strain BLS256 has 26 TAL effector genes and two apparent TAL effector pseudogenes (Bogdanove et al., 2011).

Among the 26 TAL effectors in BLS256, Tal2g has been identified as a contributor to virulence, and the biologically relevant targeted host gene is called OsSULTR3;6, which encodes a putative sulfate transporter (Cernadas et al., 2014). OsSULTR3;6 expression, as mediated by Tal2g or a dTALe, facilitates lesion expansion and bacterial exudation. Rice is predicted to contain 14 sulfate transporter genes in the cultivar Nipponbare, which were classified into five groups. OsSULTR3;6 belongs to group 3 and is expressed in uninfected rice at the later stages of panicle and seed developments. OsSULTR3;6
expression levels are very low in leaf tissue, but elevated under treatment of heavy metal chromium and drought stress (Cernadas et al., 2014; Kumar et al., 2011; Takahashi et al., 2011). In *Lotus japonica*, the most closely related gene, known as *sst1*, shares 56% identity with *OsSULTR3;6* and plays important roles in normal nodule growth and symbiotic nitrogen fixation (Krusell et al., 2005). The group 3 sulfate transporter AtSULTR3;1, AtSULTR3;2, AtSULTR3;3 and AtSULTR3;4 in *Arabidopsis* were shown to mediate sulfate assimilation in chloroplasts (Cao et al., 2013). While the chloroplast has been demonstrated to be a place where reduced sulfate is incorporated into cysteine, which indicate a potential function of these sulphate transporters in maintaining the antioxidant ability of the cytosol (Cao et al., 2013; López-Martín et al., 2008; Takahashi et al., 2011). The most similar member to OsSULTR3;6 in group 3 from *Arabidopsis* is AtSULTR3;5 (57% identity), which is otherwise localized in plasma membrane and assists another sulfate transporter AtSULTR2;1 in the root-to-shoot vascular transport of sulfate under low sulfate conditions (Kataoka et al., 2004). A hypothesis that OsSULTR3;6 promotes susceptibility through regulating redox signaling or osmotic equilibrium was proposed (Cernadas et al., 2014). The EBE of *OsSULTR3;6* is located 29 nucleotides upstream of the TATA box (Figure 1-3E) (Cernadas et al., 2014).

**S genes in bacterial spot disease of pepper**

The gene *avrBs3*, which was identified originally as an avirulence gene, is involved in the induction of hypertrophy in mesophyll cells of susceptible host plants (Bonas et al., 1989; Marois et al., 2002). The host S gene of AvrBs3 is predicted to be *upa20* (Kay et al., 2007). The gene *upa20* is comprised eight exons and seven introns and encodes a 340–amino acid basic helix-loop-helix (bHLH) transcription factor. Upa20-GFP fusion proteins were shown to localize in the nucleus, which is consistent with the predicted annotation of transcription factor. Mutations in the bHLH domain, which generally involves in DNA binding and dimerization, led to a failure to induce hypertrophy in *N.*
*benthamiana*. An *Arabidopsis* homolog BIGPETALp (BPEp) (55% identity) controls cell and, as a consequence, petal size (Szécsi et al., 2006). *Agrobacterium*-mediated transient expression of *upa20* or *avrBs3* induced enlarged palisade and spongy parenchyma cells, the typical symptom of hypertrophy in *N. benthamiana* and other solanaceous plants. However, gene silencing of *upa20* in *N. benthamiana* severely interfered the normally induced hypertrophy by AvrBs3. Further observations revealed that *upa20* expression led to cell wall invaginations and starch content decreases in chloroplasts. A cell wall modification gene α-expansin was also induced in the tissues. All the above experimental results were interpreted to hypothesize that UPA20 enhances susceptibility by regulating the pathways of cell enlargement and cell wall synthesis in response to TAL effector AvrBs3. The EBE, also called an upa-box, overlaps the TATA box of *upa20* (Figure 1-3F) (Kay et al., 2007).

**TAL effectors and E genes**

Three types of TAL effector associated R genes have been reported- recessive, dominant non-transcriptional (classical) and dominant TAL effector-dependent transcriptional based resistance. TAL effector-dependent recessive resistance occurs in rice lines with DNA polymorphisms in S gene effector binding elements and will not be discussed in detail here (Hutin et al., 2015). Dominant non-transcriptional based resistance is represented solely by the NBS-LRR resistance gene from tomato, *Bs4*, which was identified as the cognate R gene to the TAL effector gene *avrBsP/avrBs4* (Bonas *et al.*, 1993; Schornack *et al.*, 2004). However, a transcriptionally functional TAL effector is not required for *Bs4* resistance elicitation as truncated versions of the cognate avirulence gene *avrBs4* also triggers resistance. Here, we discuss the third type, namely, TAL effector dependent R genes that are both direct targets of TAL effectors in the host and identified as R genes. The genes have been referred as terminator or, here, executor R (E) genes (Bogdanove *et al.*, 2010; Tian *et al.*, 2014). E gene expression, like Avr/R gene interactions, is associated with hypersensitive response (HR) on the respective host plants and restricts
pathogen growth at the site of infection. Five E genes and the cognate TAL effector genes have been cloned, including *Xa27*, *Bs3*, *Bs4C-R*, *Xa10* and *Xa23* (Gu et al., 2005; Roemer et al., 2007; Strauss et al., 2012; Tian et al., 2014; Wang et al., 2015). The TAL effector AvrXa7 may target an as yet uncharacterized E gene *Xa7* due to the requirements for the effector nuclear localization signals and the transcription acidic activator domain in *Xa7*-dependent resistance (Hopkins et al., 1992; Yang et al., 2000).

**E proteins are not homologs of classical R proteins**

E proteins are not related on the basis of sequence to any other type of R protein. In fact, the proteins, with the exception of the recent reports on XA10 and XA23, share no sequence identity with each other. Conceptually, the E genes and their products can be divided into two groups. Group 1 consists of proteins that likely have a function in normal plants and whose function has been hijacked by host adaption to disease. Group 1 consists solely of BS3, which is a member of a conserved family of proteins known as flavin mono-oxygenases (FMO) and, more specifically, a subclass of FMOs known variously as YUCCA or FLOOZY (Figure 1-5) (Roemer et al., 2007; Exposito-Rodriguez et al., 2011; Zhao et al., 2014). Group 2 members, of which there are four, are relatively short proteins that have multiple hydrophobic potential membrane spanning domains (Figure 1-6). The proteins share no sequence relatedness with proteins of known function and the relatively few related coding sequences occur within close relatives. One related sequence outside the *Solanaceae*, from grapevine, was reported for *Bs3C-R*. Several of the E proteins may have structural similarities. XA27 and XA10 are predicted or have been shown to localize to host cellular membranes and XA10, more specifically, has been shown to localize to the endoplasmic reticulum (Wu et al., 2008; Tian et al., 2014). Prediction software also indicates that BS4C-R may be localized to the endoplasmic reticulum (Strauss et al., 2012; Nakai et al., 1999). It is tempting to speculate that BS3 requires catalytic activity for the R gene response and the
group 2 proteins function as R proteins due to their interaction with the host organelles. However, whether the predicted catalytic functions of BS3 are required for the R gene response has not been reported, and future analysis of the mechanism-of-action for the respective proteins may indicate some common feature.

**E genes in bacterial spot disease on pepper**

E genes for groups 1 and 2 have been cloned from pepper. The group 1 Bs3 is recognized by both TAL effectors AvrBs3 and AvrHah from the pathogens *X. campestris* pv. *vesicatoria* and *Xanthomonas gardneri*, respectively, both causal organisms of bacterial spot disease of pepper and tomato (Bonas et al., 1998; Schornack et al., 2008). The gene product BS3 is a 342 amino acid protein with a high degree of relatedness with FMOs (Roemer et al., 2007; Schornack et al., 2008). FMO proteins are a family of enzymes functioning in all phyla (van Berkel et al., 2006), and play roles in pathogen defense, auxin biosynthesis and metabolism of glucosinolates (Bartsch et al., 2006; Koch et al., 2006; Mishina et al., 2006; Schlaich, 2007). As noted earlier, BS3 falls in a phylogenetic clade consisting of YUCCA and ToFZY members (Figure 1-5) (Romer et al., 2007). The most closely related proteins to BS3 have been demonstrated to be involved in auxin biosynthesis and a variety of developmental and physiological responses (Exposito-Rodriguez et al., 2011; Hentrich et al., 2013; Lee et al., 2012; Stepanova et al., 2011; Zhao, 2014). YUCCA/FLOOZY members catalyze a key intermediate in the plant pathway from indole-3-pyruvate (IPA) into indole-3-acetic acid (IAA) through oxidative decarboxylation reaction (Dai et al., 2013; Hentrich et al., 2013; Kim et al., 2011; Stepanova et al., 2011; Zhao, 2014). A homologue from tomato, ToFZY, also functions in auxin biosynthesis (Exposito-Rodriguez et al., 2011). A more distant relative of unknown enzymatic activity, AtFMO1, plays a role in systemic acquired resistance (Mishina et al., 2006). Transient expression of Bs3 on pepper plants triggered an HR reaction. AvrBs3 failed to induce *Bs3*-E, an allele of *Bs3* as a result of 13-
bp nucleotide insertion in the EBE in the promoter (Figure 1-4D) (Kay et al., 2009; Roemer et al., 2007; Roemer et al., 2009b).

Bs4C-R encodes a member of group 2 E proteins and is expressed in the presence of the TAL effector AvrBs4 (Strauss et al., 2012). Bs4C-R is the only E gene isolated on the basis of differential expression between resistant and susceptible cultivars and not the typical gene mapping strategy. A two-nucleotide polymorphism in the region of the EBE of a susceptible allele Bs4C-S leads to the failure of induction of an AvrBs4-dependent HR (Figure 1-4E) (Strauss et al., 2012). Both the dominant and recessive alleles encode functionally competent proteins as constitutive expression of either Bs4C-R or Bs4C-S triggered an HR in N. benthamiana leaves (Strauss et al., 2012).

E genes in bacterial blight disease of rice

The E genes of rice are all included in our group 2 and provide resistance to bacterial blight disease. Bacterial blight of rice is caused by X. oryzae pv. oryzae, and TAL effectors are major avirulence factors for X. oryzae pv. oryzae when the cognate E genes are present in host plants (Mew, 1987; White et al., 2009). Three pairs of TAL effectors and cognate E genes have been cloned from rice -AvrXa27/Xa27, AvrXa10/Xa10, and AvrXa23/Xa23. No cognate S genes or virulence effects for the TAL effectors in compatible host cultivars have been reported for AvrXa10, AvrXa23 or AvrXa27, despite the presence of AvrXa27 and AvrXa23 in many extant strains of X. oryzae pv. oryzae (Gu et al., 2004; Wang et al., 2014).

The Xa27 product is a protein of 113 amino acids without any clear homologs based on sequence similarity in plants other than rice and several related species of the Oryza genus (Gu et al., 2005; Bimolata et al., 2013). The resistance conferred by Xa27 is affected by developmental stage, increasing with the age of the plants and reaching maximum resistance at five weeks. Moreover, Xa27 showed a
dosage effect in the cultivar CO39 genetic background (Gu et al., 2004). At least two transmembrane α-helix domains were predicted, depending on the prediction software (Gu et al., 2005). Here, we show three based on the SOSUI program (Figure 1-6, Hirokawa et al., 1998). Further experimentation has shown that the protein XA27 localizes to cytoplasmic membrane, and some protein appears in the apoplast after plasmolysis (Wu et al., 2008). Localization is dependent on the N-terminal signal anchor-like sequence, which is also essential for resistance to X. oryzae pv. oryzae (Wu et al., 2008). The protein itself appears to be toxic as gene transfer to compatible rice lines occurs with a reduced efficiency. Nevertheless recombinant lines were recovered, demonstrating that the AvrXa27-dependency of the resistance is indeed linked to the Xa27 locus (Gu et al., 2005). At the same time, lines were obtained that had elevated expression of Xa27 and displayed defense reactions, including thickened vascular elements, even in the absence of bacterial inoculation (Gu et al., 2005). The EBE is located immediately downstream of the predicted TATA box, and the recessive allele xa27 in the susceptible rice cultivar IR24 encodes the same protein but has a three-nucleotide deletion and one nucleotide difference in comparison to Xa27 (Figure 1-4A) (Roemer et al., 2009a). DNA sequence alignment of Xa27 alleles from twenty-seven lines representing four Oryza species revealed that a Xa27-related coding sequence was indeed present in all of the lines. However, only the IRBB27 allele appears to possess the necessary EBE for AvrXa27 (Bimolata et al., 2013). A synthetic TAL effector directed at the recessive allele in IR24 induced a resistance reaction, indicating the product of the recessive allele could function similarly to Xa27, if expressed (Li et al., 2013).

*Xa10* encodes a 126-amino acid protein, containing four potential transmembrane helices (Tian et al., 2014). A consensus EBE is present in the promoter region of Xa10 (Figure 1-4C). Xa10 differs from Xa27 and Bs4C-R in sequence and by the lack of a nearly identical coding sequence in susceptible plant lines. At the same time, related sequences are found in other lines, including Xa23 (Wang et al.,
Ectopic and weak expression of Xa10 in rice causes a lesion mimic-like phenotype, while transient expression of Xa10 in N. benthamiana and rice induced HR in plants (Tian et al., 2014). Under the appropriate promoter, Xa10 also induced programmed cell death (PCD) in mammalian HeLa cells (Tian et al., 2014). In both rice and N. benthamiana cells, hydrogen peroxide, swelling and degradation were detected in chloroplasts. Degradation of mitochondria was also observed, supporting the model that XA10 functions as a general inducer of PCD in plant and animal cells (Tian et al., 2014). Further functional characterization revealed that XA10 forms hexamers, localizes on the endoplasmic reticulum (ER) membrane of plant and HeLa cells, and mediates Ca^{2+} depletion, which is consistent with some processes of PCD (Pinton et al., 2008; Williams et al., 2014).

The E gene Xa23 encodes a 113-amino acid protein that shares approximately 50% amino acid sequence identity and 64% nucleotide sequence similarity with Xa10 and XA10, respectively (Wang et al., 2015). An identical recessive allele is present on the basis of the coding region, and characterization of the EBE of AvrXa23 revealed a 7-bp polymorphism accounts for the failure of xa23 induction in the susceptible rice varieties (Figure 1-4B). The susceptible cultivar JG30, with xa23, became resistant to PXO99A harboring a designed TAL effector specifically targeting the xa23 promoter region including the 7-bp polymorphism (Wang et al., 2015). Moreover, Agrobacterium-mediated transient transformation of Xa23, like Xa10, induced an HR in N. benthamiana, and also induced an HR in tomato (Wang et al., 2015). Both XA10 and XA23 have a motif of unknown function comprising of five acidic amino acid residues (EDDEE and DNDDD, respectively) at the C-termini (Tian et al., 2014). Alteration of the so-called ED motif in XA10 abolished HR activity (Tian et al., 2014).
Summary and discussion

One type of transporter genes in group 1 S genes is the SWEET gene family induced in both *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *manihotis* pathosystems (Antony et al., 2010a; Cohn et al., 2014; Yang et al., 2006; Zhou et al., 2015). Both of these two species infect their plant hosts by invading vascular tissues. Normally SWEET proteins function in regulating sugar transport (Chen et al., 2010; Chen et al., 2012). Therefore, it was hypothesized that the abilities of transporting sugars of the SWEET proteins facilitate these vascular *Xanthomonas* strains infecting their host plants by directing nutrition resources to the site of infection. However, other hypothesis should be entertained as sugars function in many signaling pathways for the host (Bolouri Moghaddam et al., 2012; Lastdrager et al., 2014). Group 1 sulfate transporter gene *OsSULTR3;6*, serves in lesion expansion and bacterial exudation in bacterial leaf streak of rice (Cernadas et al., 2014). The group 2 S genes of transcription factor genes are represented by *OsTFX1* and *upa20* (Kay et al., 2007; Sugio et al., 2007). *OsTFX1* is the target of TAL effector PthXo6 from *X. oryzae* pv. *oryzae*. However, the biological function of *OsTFX1* in disease remains cryptic (Sugio et al., 2007). Upa20 is a bHLH transcription factor and might serve in cell enlargement and inducing hypotrophy when challenged by AvrBs3 (Kay et al., 2007). One hypothesis is that hypertrophy may be part of a process that releases bacteria to the surface (Duan et al., 1999). Therefore it could be proposed that for non-vascular strains, bHLH transcription factor or sulfate transporter might facilitate bacterial exit and aggregation on the leaf surface, which is advantageous to bacterial dissemination (Cernadas et al., 2014; Kay et al., 2007a; Marois et al., 2002). Though the studies on the relationship between these S genes and their normal biological functions are few, *Xanthomonas* strains are manipulating plant normal development and physiology to enhance the susceptibility of the hosts.
Group 1 E genes consists solely of Bs3, which is the only E gene with a clear relatedness to genes of known function in uninfected plants. Again, the fact is that the protein phylogeny falls within a cluster of YUCCA family members pointing to a function of auxin biosynthesis. If catalytic, Bs3, like the S genes, may otherwise be part of the normal plant developmental process, and the catalytic function may be required for E gene function also. BS3 is analogous to the S genes in susceptibility that are hijacked for the benefit of the pathogen. E gene, however, are subverted by the host for host defense. The source and function of the group 2 E genes are less clear, and have no clear relatedness to known conserved plant genes. Group 2 E genes more likely perturb host physiology due to the structural features of the proteins and, ultimately, trigger programmed cell death. XA27, XA10, XA23 and BS4C-R have no predicted catalytic activity and no known function based on relatedness to other proteins. All are short proteins with at least two predicted transmembrane domains (Figure 1-5) (Gu et al., 2005). Weak similarity of XA10 to Ca\(^{2+}\) pores and Ca\(^{2+}\) depletion at the endoplasmic reticulum of XA10-expressing cells has been observed (Tian et al., 2014). Further characterization of the group 2 E genes may lead to insight into a common function for the encoded proteins. Regardless, the evolutionary source of the recruited E genes is not clear, and whether group 2 E genes, or Bs3, for that matter, serve any function in developmental or non-disease related physiological functions is unknown.

TAL effector genes are present in a limited number of pathogen species in comparison to some more widely spread other classes of type III effector genes, largely limited to members of the Genus *Xanthomonas* and *Ralstonia*. Nonetheless, the genes are ubiquitous in some species; have a critical function in at least one disease, namely bacterial blight of rice; apparently make major fitness contributions to a number of *Xanthomonas* diseases; and appear to be spreading into disease complexes such as bacterial spot of *solanaceous* crops (Yang et al., 2004; Cernadas et al., 2014, Hu et al., 2014;
Cohn et al., 2014; Schwartz et al., 2015). S genes, E genes and TAL effector genes facilitate interplay of the selective pressures on the host and pathogen.

Evolution exerts persistent effects on plant-pathogen interactions. Pathogen virulence conferred by major virulent TAL effectors have been defeated by several cases of recessive resistance. The recessive resistance gene xa5 encodes the γ subunit of the core transcription factor TFIIA referred as OsTFIIAγ5 (Iyer et al., 2004; Iyer-Pascuzzi et al., 2008). TFIIA is required for transcription initiation by RNA polymerase II in eukaryotes (Hampsey, 1998; Orphanides et al., 1996). Both xa5 and Xa5 are constitutively expressed in rice plants but distinct in one amino acid at position 39 (Iyer et al., 2004; Iyer-Pascuzzi et al., 2008; Jiang et al., 2006). The induction level of Os11N3/OsSWEET14 targeted by AvrXa7 was decreased when only xa5 is present in the host compared to the level when Xa5 is present. At the same time, under the background of homozygous xa5 genotype the susceptibility induced by AvrXa7 was also abolished (Antony, 2010b). Therefore, rice cultivars have acquired mutations that affect the transcription complex assembly, which attenuate TAL effector function and S gene transcription as an adaptation to bacterial disease. PthXo1 is a TAL effector whose virulence is not inhibited in homozygous xa5 plants. Expression of the S gene Os8N3/OsSWEET11 is reduced yet, apparently, sufficient to support disease (Antony, 2010b). Another TAL effector PthXo7 is able to induce OsTFIIAγ1, a paralog of OsTFIIAγ5 that might be capable to compensate partially for the dysfunction of recessive xa5 (Sugio et al., 2007).

A second type of recessive resistance is exampled by xa13, a recessive allele of Os8N3/OsSWEET11, which is not inducible by PthXo1 (Antony et al., 2010; Roemer et al., 2010; Yang et al., 2006). EBE polymorphisms account for the failed induction of xa13, an allele of OsSWEET11 by PthXo1. However, X. oryzae pv. oryzae strains can defeat the recessive allele by employing other major TAL effectors targeting alternate members in clade III of the SWEET family. A variety of TAL
effectors, including AvrXa7 and PthXo3 target an alternative SWEET gene Os11N3/ OsSWEET14 to accomplish bacterial pathogenicity, while yet another, PthXo2, targets OsSWEET13 (Antony et al., 2010; Chu et al., 2006; Streubel et al., 2013; Yang et al., 2006; Yu et al., 2011; Zhou et al., 2015). The same principle should extend to other TAL effectors and the respective pathogens, though there is no evidence outside of bacterial blight disease of rice currently.

The E genes and their products stand as a mode of host adaptation to TAL effectors, foiling the pathogens with the very TAL effector mode-of-action in virulence. The difference between dominant and recessive E gene alleles indicates that host plants acquire TAL effector-mediated resistance through nucleotide polymorphisms in the corresponding promoter regions to recognize the cognate TAL effectors. Yet, again, the relatively small number of E gene examples leaves open to question the selective pressures for E gene selection. Only one of the presently known alleles of Xa27, for example, possessed an apparent AvrXa27 binding site and, therefore, the alleles do not appear to be directed toward the AvrXa27, which is present in many extant Asian strains (Gu et al., 2004; Bimolata et al., 2013). On the other hand, bacteria can rapidly evolve to avoid the recognition through gene loss under high selection pressure (Koskiniemi et al., 2012). For TAL effectors, loss of a single repeat can lead to E gene failure. Deletion of repeats in AvrBs3, for example, resulted in the loss of the induction of Bs3 (Herbers et al., 1992; Kay et al., 2009). Deployment of an E gene that targets a critical TAL effector for virulence in bacterial blight disease would seem to make adaption of the bacterial population less likely as the pathogen would have to maintain virulence in addition to losing Xa7 recognition. Indeed, Xa7, which perceives the major TAL effector AvrXa7, was found to be durable for at least ten years in test fields in the Philippines (Cruz et al., 2000). Nonetheless, in vitro rearrangements and in vivo selection for loss of AvrXa7-mediated resistance readily produced forms that maintained virulence and avoided Xa7-mediated resistance (Yang et al., 2005). In addition, a variety of TAL effectors target the AvrXa7
target, OsSWEET14, without Xa7-dependent resistance activity (Antony et al., 2010a; Streubel et al., 2013), and, in India, field strain surveys have found a diversity of strains, many without AvrXa7 activity (Mishra et al., 2013). Thus, broad application of a single E gene like Xa7 appears to be limited, although local conditions, such as the Philippine tests, may limit the invasion of a particular TAL effector gene into extant pathogen populations (Cruz et al., 2000).

Discovering the diversified TAL effector induced S and E genes will not only add new knowledge to understand the evolutionary pathways in the pathogen and host interactions, but also provide new tools to control the disease. Resistant rice lines have been developed by inducing changes in the EBE of one S gene Os11N3/OsSWEET14 using the specific designed TAL effector nucleases (TALENs), by which the recognition sites for the cognate TAL effectors were mutated and hence the virulence function induced by AvrXa7 or PthXo3 was interrupted without interfering the developmental function of it (Li et al., 2012b). The development of the facile genome editing tool of CRISPR/Cas9 may allow ready modification of EBEs of S genes and improve the tools for breeding resistant crop varieties to Xanthomonas/TAL effector associated diseases (Belhaj et al., 2015; Bortesi et al., 2015; Zhou et al., 2015). E genes can be employed with hybrid so-called super-promoters, consisting of multiple EBEs, each recognizing specific corresponding TAL effectors that are expressed by the pathogen populations (Roemer et al., 2009a; Hummel et al., 2012; Zeng et al., 2015). Xa27 was fused to a super promoter including binding sites for three TAL effectors from X. oryzae pv. oryzae and three from the bacterial leaf streak pathogen X. oryzae pv. oryzicola. The plants were resistant to several X. oryzae pv. oryzae and X. oryzae pv. oryzicola strains that were originally compatible on wild type homozygous Xa27 plants (Hummel et al., 2012). Similarly, transgenic rice lines containing Xa10E5 with binding elements to five TAL effectors were proved to be resistant to 27 of the 28 X. oryzae pv. oryzae strains gathered from 11 countries (Zeng et al., 2015). Therefore, judicial choices of the EBEs for TAL effectors that are
expressed by the extant populations may provide resilient barriers to bacterial blight and related diseases in other crop species.

Relatively few S genes that are associated with TAL effector genes in *Xanthomonas* have been identified. Though other disease systems are out there await to be studied such as bacterial blight of cotton and citrus bacterial canker diseases in which TAL effectors have been evidenced as major virulence factors (Al-Saadi et al., 2007; Swarup et al., 1992; Yang et al., 1994; Yang et al., 1995). However, the cognate targets remain to be illustrated. Also some other pathosystems like bacterial leaf streak of wheat, bacterial alfalfa leaf spot, soybean bacterial pustule diseases, the contribution of TAL effectors to bacterial virulence are still unknown. My project is to explore more S genes in two disease systems including citrus bacterial canker and soybean bacterial pustule diseases, which will refine our understanding of the mechanisms of TAL effector mediated pathogenesis on hosts, especially in the aspect of non-vascular *Xanthomonas* involved pathosystems.
Figure 1-1 The structure of a prototypic TAL effector.

A schematic of a typical TAL effector, indicating the N-terminal translocation domain, 3 NLS (nuclear localization signal) sites, and the AD (acidic transcription activation domain). The repeat region is represented by part of the repeat region of AvrBs3, the letters in each repeats are amino acids. The red letters are RVDs.

Figure 1-2 The code for common RVDs and the associated nucleotides.

The above code is based on the work of Grau et al (Grau et al., 2013). Briefly, each RVD has been found to be associated preferentially with one or more of the four nucleotides. The larger the font of the nucleotide, the greater probability of the association with the nucleotide.
Figure 1-3 Promoters of S genes and nucleotide polymorphisms in dominant and recessive alleles.

(A) Sequence alignment of the partial promoter regions of Xa13 (the dominant Os8N3) from the rice cultivar IR24 (gi|89892339|gb|DQ421396.1)|1 and the xa13 promoter from the rice cultivar IRBB13 (gi|89892335|gb|DQ421394.1)|1. (B) Sequence of the partial promoter regions of rice Os11N3/OsSWEET14 (Os11g31190)|2. (C) OsTFX1 (Os09g0474000)|2. (D) MeSWEET10a from cassava (Manihot esculenta) (cassava4.1_013474)|3. (E) OsSULTR3;6 (Os01g52130)|2. (F) upa20 from pepper (Capsicum annuum) (gi|158147059|gb|EU046276.1)|1. The ATG start codon is displayed in red letters. Nucleotides that are identical between the alleles are displayed as black letters. The predicted TATA boxes are highlighted by underlines. The yellow color highlights the EBEs with blue letters indicating the difference between the alleles. TAL effectors are represented by the repeat regions in which a single letter indicates a RVD (I-NI; G-NG, HG or YG; S-NS; D-HD or ND; *-N*, N-NN or SN).

1NCBI ID

2Locus ID from RiceGE: Rice Functional Genomic Express Database

3Phytozome ID
**A**

AvrXa27-IN*GSNNNINI*DDIGG

Xa27 TATAAA TAGAAGAAGAGACCTATA GAGAGCATCAGAGCAAAGTACTCCCTAAAAGACAGCCACACACACTGAG

xa27 TATAAA TAGAAGA---GACCAATA GAGAGCATCAGAGCAAAGTACTCCCTAAAAGACAGCCACACACACTGAG

Xa27 ACACCAAGAAGCTGCTCTCAAATG

xa27 ACACCAAGAAGCTGCTCTCAAATG

**B**

AvrXa23-DDN------NGGSGDG*DDD*NINDDDNG*  

Xa23 GCTACTTAAAAAGTCCTCTTCCG--------AAACAT CTTCCTCCCCGCTACAATACAGTTCTCTATA

Xa23 GCTACTTAAAAAGTCCTCTTCCGCTACAATACAGTTCTCTCTATA

Xa23 AAAGCCCTTCTCTGTAAGTACTCTCAAGGAGCTGCAAGCATCTTCTCTGACACTCCTCTCTCAT

Xa23 AAAGCCCTTCTCTGTAAGTACTCTCAAGGAGCTGCAAGCATCTTCTCTGACACTCCTCTCTCAT

**C**

Xa23 CTCAAGGAGTTGCAAAATGTT

Xa23 CTCAAGGAGTTGCAAAATGTT

**D**

AvrXa10-IGIGIINDIDNGSGD*

Xa10 TCACGTTCACTCTCTTTA TATATACACAGTTGCACT CTCTCTTTATATACAGTTCTCTCTCTACTTCA

Xa10 TTCTTCATTAGCAGGACAGAGTCGAGAAGCTTCCCTCCCTCCCTCATTCTCTCTGAGATG

AvrBs3-DGSGIIIDDG----------------SSDDDG

**E**

Bs3 TGCCTGACCAATTTTAT TATATACACAGTTGCACT CTCTCTTTATATACAGTTCTCTCTCTACTTCA

Bs3-E TGCCTGACCAATTTTAT TATATACACAGTTGCACT CTCTCTTTATATACAGTTCTCTCTCTACTTCA

Bs3 CCTTTCCTTTTCCTCTTTGTTCTGTGTCACCCGCTAATCATTCAAAACAGTACTCTCTTTAGTGTCACA

Bs3-E CCTTTCCTTTTCCTCTTTGTTCTGTGTCACCCGCTAATCATTCAAAACAGTACTCTCTTTAGTGTCACA

Bs3 TATATTCATG

Bs3-E TATATTCATG
Figure 1-4 Promoter of E genes and polymorphisms in dominant and recessive alleles.

(A) Sequence alignment of the partial promoter regions of Xa27 from the rice cultivar IRBB27 (gi 66735941 gb AY986491.1) and the xa27 promoter from the rice cultivar IR24 (gi 66735943 gb AY986492.1). (B) Sequence of the partial promoter regions of rice Xa10 from IRBB10 (gi|448280729|gb|JX025645.1). (C) Xa23 from the rice cultivar CBB23 (gi|721363841|gb|KP123634.1) and the xa23 promoter from JG30 (gi|721363854|gb|KP123635.1). (D) Bs3 from Capsicum annuum cultivar ECW-30R (gi|158851516|gb|EU078684.1) and the Bs3-E promoter from C. annuum cultivar ECW (gi|158851512|gb|EU078683.1). (E) Bs4C-R from C. pubescens cultivar PI 235047 (gi|414148024|gb|JX944826.1) and the Bs4C-S promoter from C. pubescens cultivar PI 585270(gi|414148026|gb|JX944827.1). The ATG start codon is displayed in red letters. Nucleotides that are identical between the alleles are displayed as black letters. The predicted TATA boxes are highlighted by underlines. The yellow color highlights the EBEs with blue letters indicating the difference between the alleles. The TAL effectors are represented by the repeat regions in which a single letter indicates a RVD (I-NI; G-NG or HG; S-NS; D-HD or ND, *-N*, N-NN).
Figure 1-5 A phylogenetic tree of BS3 related proteins.

Proteins from closely related YUCCA genes of \textit{C. annuum} (Ca), \textit{A. thaliana} (At), Tomato (To), \textit{C. sinensis} (Cs) and the predicted pepper (Ca) BS3 (Capana02g001306) protein were aligned. Names of proteins are given with the Phytozome ID or Pepper Genome Database ID (in parentheses). A monophyletic group that contains the predicted BS3 protein and tomato YUCCA-like proteins is boxed. Sequences are aligned with the online ClustalW server (http://www.ch.embnet.org/software/ClustalW.html) using the default values. MEGA6.0 was used for generating a tree on the basis of ClustalW output. Phylogenetic calculations are based on the maximum likelihood method, and Bootstrap analysis was used to evaluate the reliability of the nodes of the phylogenetic trees. Bootstrap values are based on 1000 replications. The branch lengths of the tree are proportional to divergence. The 0.1 scale represents 10% change.
**Figure 1-6 Structural predictions for group 2 E proteins.**

A. Bs4C-R; B. XA27; C. XA10 and XA23. Alignment of XA10 and XA23 using ClustalW2 with the default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The yellow color highlights the predicted transmembrane helices using the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html).
Chapter 2 - Functional analysis of TAL effector PthAw in citrus bacterial canker

Abstract


Citrus bacterial canker (CBC) disease is an economically important disease worldwide on all kinds of citrus varieties. The causal pathogens of CBC are members of the genus *Xanthomonas*. Five groups (group A, group A*, group Aw, group B, and group C) of pathovars have been identified. TAL effectors as a part of T3S effectors are an important group of virulence factors in the CBC system. *PthA* and other *pthA* homologous genes identified from the five groups of citrus canker causal strains of *Xanthomonas, pthA4, pthAw, pthA*, pthB and pthC* were shown to determine the virulence of individual pathovars. By using microarray assay and designed TAL effectors (dTALes), we identified that *CsLOB1*, a members of the *lateral organ boundaries domain* (*LBD*) gene family is an S gene targeted by PthAw and other multiple major TAL effectors from CBC causal strains.

Introduction

Citrus bacterial canker (CBC) disease is an economically important disease worldwide on all kinds of citrus varieties including sweet orange, grapefruit, Mexican limes and others. The bacteria infection begins by entering into the citrus plants through stomata and wounds and colonizing the apoplast. An erumpent pustule is visible within six to eight days at the site of infection. The typical symptom is the raised erumpent pustules on leaves, stems and fruits. Lesions also have water-soaked and yellow halos. Severe infection can cause defoliation and premature fruit drop (N. Gottig et al., 2010). The disease is very persistent once established in an area. In North America, the first outbreak happened in Florida and adjacent states in 1912, and was declared being eradicated in 1933. The second
occurrence of CBC was in 1986 in Florida, and was claimed being removed in 1994. The disease re-emerged on Miami residential citrus trees in 1995. Eradication as an effective disease control measure has been abandoned, and CBC can be detected in both residential areas and commercial groves (Gottwald et al., 2002). The economic impact of CBC on the citrus industry in Florida has been intense.

The causal pathogens of CBC are members of the genus *Xanthomonas*. Five groups (group A, group A*, group Aw, group B, and group C) of pathovars have been identified. Groups A, A* and Aw (XccA, XccA* and XccAw) are designated into the species of *Xanthomonas citri* subspecies (ssp.) *citri* (Xcc) (syn. *X. axonopodis pv. citri*, *X. campestris pv. citri*, *X. citri pv. citri*) originated from Asia, while group B and C (XauB and XauC) originated from South America belong to species of *X. fuscans* ssp. *aurantifolii* (Xau) (Al-Saadi et al., 2007; Sun et al., 2004; Verniere et al., 1998). Group A has the broadest host range and is the most widely spread and virulent pathotype. Group B are pathogenic on lemon (*C. limon*), Mexican lime (*C. aurantifolia*), sweet orange (*C. sinensis*) and grapefruit (*C. paradisi*). XccAw strains cause canker on Mexican lime and alemow (*C. macrophyla*). Group A* and group C can only infect Mexican lime. Grapefruit (*C. paradisi*) is highly susceptible to Xcc group A strains, but is resistant to group A*, Aw, and C strains (Al-Saadi et al., 2007; Sun et al., 2004; Verniere et al., 1998). Phylogenetic trees constructed using the *Xanthomonas* conserved genes showed that XccAw and XccA are in one clade, and XauB and XauC are in a separate clade (Jalan et al., 2013).

Bacterial attachment to the plant tissues is the initial stage for CBC. Attachment is mediated by adhesins located on the bacterial cell surface and exported by type V protein secretion system (N. Gottig et al., 2010). The Xcc adhesion gene XacFhaB had been shown important in bacterial virulence. A mutation in xacFhaB reduced the number and the size of cankers (Gottig et al., 2009). (The nomenclature of Xcc strains and, consequently, the strain acronyms have changed repeatedly, giving rise to Xcc/Xac variations). Xanthan gum is the featured exopolysaccharide product of genus *Xanthomonas*.
and was shown to be able to facilitate the bacterial epiphytic survival and protect the pathogen under adverse condition (Dunger et al., 2007). A bacterial biofilm is a structure composed of bacterial cells and the surrounding self-produced polymeric matrix, and adheres to static or living surfaces. A XacFhaB adhesin and the exopolysacharide xanthan are both involved in formation of Xcc biofilms, improving the bacterial epiphytic survival on host tissues, and facilitating the colonization of more distant tissues and disease development in the later stages of CBC (Gottig et al., 2009).

Lipopolisaccharide (LPS) is an essential and conserved cell surface component of Gram negative bacteria (Proteobacteria) and plays an important role in bacterial virulence in CBC. Two Xcc LPS biosynthesis genes (wzt and rfb303) were shown to function in bacterial resistance to external stresses, in bacterial movement, in \textit{in vivo} and \textit{in vitro} adhesion and in biofilm formation. Mutations in these two genes reduced the bacterial virulence and decreased the induction of the host plant defense-related genes (Petrocelli et al., 2012). Recently, a putative glycosyltransferase gene \textit{gpsX} was reported to be involved in EPS and LPS synthesis and biofilm formation in Xcc, and to be essential for Xcc full virulence on host plants (Li et al., 2012a).

The type II secretion system (T2SS) is a protein secreting system transporting toxins and various hydrolytic enzymes to degrade the cell wall in plant hosts (Buettner et al., 2010). Mutations in T2SS (\textit{xpsD}, \textit{xpsM}, \textit{xpsN}, \textit{xpsE}, \textit{xpsF}, and \textit{xpsG}) showed a delayed symptom development on citrus leaves (Yan et al., 2012). Another specific virulence factor of Xcc is a plant natriuretic peptide (PNP)-like protein, which regulate the host homeostasis, including stomatal opening and photosynthesis. The mutation on gene encoding the XacPNP caused large necrotic areas. However, when the mutant strain was complemented with the XacPNP gene, or the mutant strain co-infiltrated with the purified XacPNP protein, the necrotic areas were reduced (Garavaglia et al., 2010; Gottig et al., 2008).
When the host plants sense bacterial PAMPs, such as flagellin and elongation factor Tu (EF-Tu) through the pathogen recognition receptors (PRRs) on the plant cell surface, the PAMP-triggered immune (PTI) responses will be induced to inhibit the infection of the bacteria. To overcome this barrier of plant defense, type III secretion (T3S) effectors are employed by bacteria to enhance their virulence and disease symptomatology (N. Gottig et al., 2010). T3S effectors are secreted by T3S system, which are conserved in all the Xanthomonas species. T3S effectors of Xanthomonas species have diversified biological functions. For example, the two T3S effectors from X. campestris pv. vesicatoria XopD and XopN have been reported as virulence effectors in disease symptomatology in tomato. XopD contains a small ubiquitin-like modifier (SUMO) protease domain, which regulates protein stability by SUMOylation of host proteins. XopN has been shown to interact with a receptor-linked kinase named TARK1 in suppressing host plant defense reaction (J. Kim et al., 2008; J. Kim et al., 2009; White et al., 2009a).

Comparative genomic analyses of xanthomonads have uncovered the T3S effector gene repertoires in CBC strains. Twelve effector genes (xopA, xopE1, xopE3, pthA4 or functional homologs, xopI, xopV, xopAD, xopAI, xopAK, xopAP, hpaA, and hrpW) can be found in all four CBC causing groups (Xcaw, XccA, XauB and XauC). The genes xopAQ, xopE2, xopN, xopP and xopAE are not present in XauC strains and are present in Xcaw12879, XccA306 and XauB. Also xopB, xopE4 and xopJ1 can be found in both XauB and XauC strains but are absent in XccA306 and Xcaw12879. XopC1 and xapAG are present in some A* strains. Effector genes xopAF and xopAG/avrGf1 were only present in Xcaw, XauB and XauC but not in XccA306. XopAF helps to increase the bacteria populations on grapefruit and Mexican lime plants. AvrGf1 induces HR on grapefruit plants (Rybak et al., 2009). In XauB, xopAG gene is truncated by a transposon, which may explains why XauB strains can infect grapefruit (Escalon et al., 2013; Jalan et al., 2013; Moreira et al., 2010).
TAL effectors as a part of T3S effectors are another important group of virulence factors in the CBC system. PthA from group A is the first identified member of TAL effectors demonstrated to function in pathogenicity (Swarup et al., 1992). Later, other pthA homologous genes were determined from the five groups of citrus canker causal strains of Xanthomonas, they are pthA4, pthAw, pthA*, pthB and pthC, and all of them determine the virulence of individual pathovars (Al-Saadi et al., 2007). TAL effectors function as host transcription factors and the targeted genes in rice and pepper have been identified (Bock et al., 2014). For examples, OsSWEET11, OsSWEET13 and OsSWEET14 were shown to be the S genes targeted by a variety of TAL effectors from X. oryzae pv. oryzae in rice bacterial blight (Antony et al., 2010a; Yang et al., 2006; Zhou et al., 2015). The upa20 gene, encoding a bHLH transcription factor, is the S gene in pepper bacterial leaf spot targeted by TAL effector AvrBs3 (Kay et al., 2007). The cognate S gene or genes, if any, of the TAL effectors of CBC causal pathogens are unknown. Here, we used transcription profiling to identify an S gene targeted by PthAw and other multiple major TAL effectors from CBC causal strains.

**Methods and materials**

**Plant material, bacterial strains, and plasmids**

Grapefruit trees were grown at 28°C, 80% humidity, at a setting for 16 hour day and 8 hour dark. All Xanthomonas strains were incubated at 28°C, and E.coli strains were cultured at 37°C. All the bacterial strains and plasmids are shown in Appendix A-1. E. coli competent cells used were Stellar™ Competent Cells from Clontech or NEB 5-alpha Competent E. coli (High Efficiency) from New England Biology (NEB). The Xanthomonas competent cells were made by washing the cultured bacteria with 10% glycerol three times, and transformed using electroporation method (Alexandre M Do et al., 2005).
Bacterium inoculation

*Xanthomonas* strains were streaked on TSA media, incubated at 28°C for 48 hours. Single colonies were picked, spread on new TSA plates, and incubated at 28°C for two days. The bacteria were suspended in sterilized double distilled water to OD_{600}=0.5 and, where required, the suspensions were diluted into 10^3X in double distilled water. The bacterial suspensions were delivered into the lower side of young grapefruit leaves with a needleless 1ml syringe.

**Designed TAL effector construction**

Four types of repeats (NI, NN, NG, and HD) that correspond to the respective nucleotides A, G, T, and C were used to assemble the middle repeat domains of the artificial dTALes according to TAL effector assembly method of Ting Li’s (Li et al., 2011). Restriction enzymes of *BstAPI* and *AatII* were used to replace the repeat region of TAL effector gene *pthAw*. Each gene was sequenced to verify each construct.

**RNA extraction, reverse transcription and quantitative RT-PCR analysis**

Grapefruit leaves were syringe-infiltrated with bacterial suspensions at OD_{600}=0.5, and the leaf tissues were harvested at 5 days post inoculation (dpi). Total RNA was extracted using TRIzol Reagent (Life Technology) following the manufacturer’s instruction. The RNA was subjected to LiCl re-precipitation and DNase treatment to remove the remaining DNA. First-strand cDNA synthesis was achieved using Verso cDNA Synthesis Kit (Thermo Scientific). Two-step real-time PCR was performed using iQ™ SYBR® Green Supermix (BIO-RAD). The gene-specific primer sequences are listed in Appendix A-2. The gene with transcript ID number of orange1.1g001725m (Phytozome) was used as endogenous control. The 2^{-ΔΔCt} method was used for relative quantification (Livak et al., 2001).
**Microarray assays**

Grapefruit leaves with the respective inoculations were harvested at 5 dpi when the pustules start to appear on grapefruit leaves. The 5 dpi time point was chosen to allow time for the induction of the bacterial type III secretion system and just prior to the appearance of visible pustule symptoms. For each strain, three replications were performed. GeneChip Citrus Genome Array (Affymetrix) was used to monitor the gene expression. The microarray assay and initial data analysis were conducted by the K-State Integrated Genomics Facility (IGF). Gene annotations were retrieved from PLEXdb [http://www.plexdb.org/]. T-tests were used for statistical analyses.

**Candidate gene retrieval and promoter analysis**

The probe DNA sequences retrieved from PLEXdb were used to blast the phytozome *C. sinensis* database. The gene with the highest similarity for each probe was chosen for promoter analysis. The 1000bp upstream DNA sequences from the initiation codon ATG were scanned for putative EBEs for TAL effector PthAw using TALgetter web-server (Grau et al., 2013).

**Thin sectioning**

The inoculated plant leaves were cut into smaller pieces (~1mm) and fixed with 6% glutaraldehyde in PBS buffer for 24h at room temperature. During this time, the fixative was changed three times. The samples were rinsed three times in PBS buffer, and post fixed overnight with 2% OsO4 in the same buffer. The specimens were rinsed in PBS buffer, dehydrated with 30%, 50%, 70%, 80%, 90%, 95% ethyl alcohol, each one for 10 minutes respectively and in 100% EtOH twice for 10 minutes and embedded in 812 Epon resin. The embedded specimens were sliced into 2 micron thin sections using an ultramicrotome (Leica EM UC6, Germany) and stained in Trypon Blue for 1h before imaging in epi-fluorescent upright Microscope (Zeiss AXIO PLAN2, Germany) using a digital CCD camera.
Bacterial population assay in citrus plants

Each strain was inoculated at the concentration of $10^3$X dilution of the bacterial suspension solution of OD$_{600}$=0.5. Six pieces of 1 cm$^2$ leaf disk in inoculation area for each treatment was taken and grounded in 10ml sterile water, after serial dilutions, 50 ul were plated on TSA medium and incubated at 28 °C for 2 d. The colony number was counted to determine the internal populations. Each experiment was repeated three times.

Results

PthAw is a virulence effector in Xcc306

To test whether PthAw is a virulence effector for CBC in strain Xcc306, specifically, a PthA4 mutant strain Xcc306ΔpthA4 was used as a receptor to generate a TAL effector gene pthAw complemented strain Xcc306ΔpthA4::pthAw. Water (mock), Xcc306WT, Xcc306ΔpthA4, Xcc306ΔpthA4::pthAw and T3S system mutant Xcc306HrcC- were inoculated on young lower sides of grapefruit leaves. Xcc306ΔpthA4::pthAw caused typical CBC symptom of pustule formation similarly to Xcc306WT (Figure 2-1II and IV). The sites infected with strain of Xcc306ΔpthA4 also showed bacterial spread, but no pustule formation was evident (Figure 2-1III). Xcc306HrcC-, which is defective for T3S system-mediated secretion, showed no disease development (Figure 2-1V).

Identification of the candidate targeted S gene of PthAw using microarray assay

In order to identify candidate targeted S genes of PthAw, Affymetrix microarray assays of RNA samples of grapefruit leaves with three treatments of Xcc306ΔpthA4::pthAw, Xcc306ΔpthA4 and Xcc306HrcC- were conducted. Two comparisons, Xcc306ΔpthA4::pthAw vs. Xcc306ΔpthA4 and
Xcc306ΔpthA4::pthAw vs. Xcc306HrcC, were made. Firstly, the probes with fold changes over six for the two comparisons were chosen for annotation and promoter analysis. However if candidates could not be discovered in the set range of fold change of six, then a new fold change range limit was reset to a lower value, and do the annotation and promoter analysis. The same procedure continued until candidates were exposed. Fortunately, two candidates were discovered from the first attempt of searching and in the list of top seven up regulated ones. The probe IDs, annotations and the fold changes of the top seven candidates are listed in Table 2-1. The fold changes of all top 7 probes in both two comparisons were significant ($P < 0.05$). The probe ID numbers for the two candidates are Cit.3027.1.S1_s_at and Cit.37210.1.S1_at whose DNA sequences were obtained from PLEXdb and were used as queries to blast the genome database of *C. sinensis* (Phytozome http://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST). Transcripts *orange1.1g025686m* and *orange1.1g026556m* were achieved for Cit.3027.1.S1_s_at and Cit.37210.1.S1_at respectively. *Orange1.1g025686m* is annotated as a SWEET gene whose homologs have been identified as the S genes in rice bacterial blight disease containing a predicted EBE in the promoter with score of -19.55 (Figure 2-2) (TALgetter web-server). *Orange1.1g026556m* annotated as a member of the family of *lateral organ boundary domain* (LBD) genes contains a predicted EBE for PthAw in the promoter with score of -13 (Figure 2-2) (TALgetter web-server). Further qRT-PCR results confirmed that the elevated expression of the two genes was associated with the presence of PthAw (Figure 2-3). Hereafter, these two putative S genes *orange1.1g025686m* and *orange1.1g026556m* were referred to as CsSWEET1 and CsLOB1, respectively.

**Validation of the S genes using designed TAL effectors**

To test the functional requirements of CsSWEET1 and/or CsLOB1 as the S genes in CBC, designed TAL effectors (dTALes) with optimized repeats corresponding to the specific EBEs in
promoters of *CsSWEET1* and *CsLOB1* were generated (Figure 2-2, squared nucleotides). The rationale is that dTALes can discriminate between S genes and off-target genes. The dTALes with the optimized repeats will specifically induce the targeted gene and are not likely to include the same set of off-target genes. After introduction of dTALes dCsSWEET1 and dCsLOB1 into Xcc306ΔpthA4, the targeted genes CsSWEET1 and CsLOB1 were demonstrated to be induced by the respective dTALes (Figure 2-3A). However, only dCsLOB1, but not dCsSWEET1, complemented the low virulence of Xcc306ΔpthA4 in terms of pustule formation, cell hyperplasia and bacterial proliferation (Figure 2-3A, B and Figure 2-4).

The promoter of S gene CsLOB1 contains EBEs for multiple TAL effectors in CBC

Based on TALgetter (TAL effector target site prediction software), four additional TAL effectors from Xcc and Xau (PthA4, PthA*, PthB and PthC) have potential EBEs in the *CsLOB1* promoter. The only difference is that the EBE for PthB and PthC is 6-bp upstream of the ones for PthA4, PthAw and PthA* (Figure2-5).

Discussion

Although both *CsSWEET1* and *CsLOB1* can be induced in the presence of TAL effector PthAw, the results indicates that only *CsLOB1* functions in pustule formation and enhancing bacterial populations after each gene was targeted by unique dTALes. In similar to upa20 and OsTFX1, CsLOB1 is a member of a transcription factor family (Kay et al., 2007; Sugio et al., 2007). An Arabdopsis LOB has been shown to be capable of interacting with members of the bHLH family, and the binding of bHLH048 to LOB led to a reduction of LOB affinity to DNA (Husbands et al., 2007). CsLOB1 may therefore collaborate with citrus bHLH members to control the same downstream pathways. Therefore, Pepper bacterial leaf spot, which involves the induction of bHLH gene upa20, and CBC causal strains might employ genes within a similar regulatory pathway to promote diseases on the respective hosts.
Although not correlated with the formation of pustules, CsSWEET1 expression may play a role in the fitness of pathogens in field conditions. In the case of rice bacterial blight, the three S genes OsSWEET11, OsSWEET13 and OsSWEET14 were shown to be sucrose transporter and the sucrose transporters were proposed to provide nutrition to bacteria and facilitate the bacterial infection (Antony et al., 2010a; Chen et al., 2010; Chen et al., 2012; Yang et al., 2006; Zhou et al., 2015). CsSWEET1 was capable of transporting both sucrose and glucose (Hu et al., 2014). However, the gene encodes a member that has a greater preference for glucose, and more work is required to clarify the biological contribution of CsSWEET1 to CBC.

Understanding the TAL effector targets involved in CBC should allow the engineering of TAL effector insensitive varieties. Resistant cultivars in rice have been obtained by selecting host cultivars with defective S genes associated with the major TAL effectors of the pathogen (Chu et al., 2006; Yang et al., 2006). More recently, cultivars with defective S genes can be constructed using CRISPR/Cas9 or TAL effector nuclease (TALEN) technologies. One case has been accomplished in rice. The EBEs in the promoter of the S gene OsSWEET14/Os11N3 was mutated using the TALEN and hence the virulence function induced by AvrXa7 or PthXo3 was interrupted without interfering the developmental function of it (Li et al., 2012b). CRISPR/Cas9 strategy has also been widely used for genome editing on various plant species (Belhaj et al., 2015; Bortesi et al., 2015). OsSWEET13 knockout mutants generated by editing the coding region using CRISPR technique could not confer the susceptibility status when challenged by X. oryzae pv. oryzae strain containing the cognate TAL effector PthXo2 without effect on plant morphology (Zhou et al., 2015). Similar approaches should be possible by targeting the promoter of CsLOB1.

The second option is to engineer citrus R genes using the cognate EBEs of the various Xcc and Xau TAL effectors. CBC resistance genes can be identified by designing dTALes targeting citrus genes
that are homologous to the characterized R genes in other plants, such as *Bs3, Xa10* and *Xa21*.

Furthermore, the super promoter identified here can be used as a promoter-trap inserted in the promoters of the R genes that will be induced by most CBC causal strains. By pyramiding the two strategies of silencing the S gene and over-expressing the R genes, broad spectrum, durable and pathogen-adapted resistant varieties could be generated to defeat CBC.
Figure 2-1 Pustule formation induced by the Xcc306 strains carrying PthAw effector.
Grapefruit leaves inoculated with I: H₂O; II: Xcc306WT; III: Xcc306ΔpthA4; IV: Xcc306ΔpthA4::pthAw; V: Xcc306HrcC. Pictures were taken at 7dpi, inoculum was OD₆₀₀=0.5.

**PthAw**-IGGSDSDDGCGGSDGG

**A**

\[
\text{TCCCTAGTTTCTTTGAAATCCCTATAVAAACCCCTTTGCTTGCTACCC} \\
\text{ACAAGTCATTHTGCATTTCACAAAAGAAACTCGTGGTTTTCTATTTCTTGAGTT} \\
\text{CTTTCCCTCATCGAGTTCTCTTTCCCTTCTTCACTGAA}
\]

**B**

\[
\text{ATTGTCATTCTTGCCTTTTCTCTCTATATAAACCCCTTTGCTTAACTTTT} \\
\text{TTCAACTAAAGCAGCTCCCTCTCATCCCTTACTGCTTCTGCGTTTTCTCTACTAC} \\
\text{TACAAACCCACAGTTTTCTCTCTCAAAAAATGGAATGCGAGACACAAATTAATGTA} \\
\text{GCAATCCCAAATCACAAT}
\]

Figure 2-2 Potential EBEs in promoters of orange1.1g025686m and orange1.1g026556m

EBE analysis of *CsSWEET1* (orange1.1g025686m) (A) and *CsLOB1* (orange1.1g026556m) (B) for PthAw. PthAw is represented by the repeat regions in which a single letter indicates an individual RVD (I=NI; G=NG; S=NS; D=HD, *=*N*). The RVDs are aligned with the predicted EBEs in the promoter region. Red letters indicate the optimal match between the RVDs and the corresponding nucleotides. The black squares in A and B enclose the binding sites for dTALes for *CsSWEET1* and *CsLOB1* respectively.
Figure 2-3 Validation of induction of CsLOB1 and CsSWEET1 by PthAw.

QRT-PCR of mRNA of grapefruit leaves inoculated with Xcc306ΔpthA4 and Xcc306ΔpthA4::pthAw. Samples were taken at 5dpi. * indicates significant difference of the gene induction level by Xcc306ΔpthA4::pthAw relative to Xcc306ΔpthA4 (t-test P < 0.05).
Figure 2-4 Pustule formation is dependent on CsLOB1.

A. Semi-quantitative PCR of mRNA from grapefruit leaves inoculated with various strains as labeled at the bottom of each gel picture. The gene names are to the right of the PCR products in each panel. CD575345.1 is a housekeeping gene serving as a control gene in the semi-quantitative PCR reaction.

B. Symptoms on grapefruit leaves induced by various strains. C. Thin sections of grapefruit leaves inoculated with various strains. (I: Xcc306ΔpthA4::pthAw; II: Xcc306ΔpthA4::dCsLOB1; III: Xcc306ΔpthA4::dCsSWEET1; IV: Xcc306ΔpthA4).
Figure 2-5 In planta growth of Xcc306ΔpthA4 mutant (square) and the corresponding complemented strains.

Sweet orange leaves were inoculated at the concentration of 5×10^5 cfu/ml (10^3X dilution of bacterial suspension of OD_{600}=0.5), the population was monitored at the time points as indicated. Error bars represent standard deviations. The experiment was repeated three times with similar results. Different letters indicate significant difference at level of P < 0.05 (t-test).

**CsLOB1**

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PthB</td>
<td>8</td>
</tr>
<tr>
<td>PthC</td>
<td>11</td>
</tr>
<tr>
<td>PthA*</td>
<td>-18</td>
</tr>
<tr>
<td>PthAW</td>
<td>-13</td>
</tr>
<tr>
<td>PthA4</td>
<td>-11</td>
</tr>
</tbody>
</table>

Figure 2-6 EBE predictions of the five TAL effectors on promoter of CsLOB1.

The number is the score returned by the model from TALgetter web-server (Grau et al., 2013)
### Table 2-1 Top seven PthAw up-regulated genes.

<table>
<thead>
<tr>
<th>Affymetrix ID Probe</th>
<th>Gene Annotation</th>
<th>Fold change</th>
<th>Xcc306ΔpthA4::pthAw vs. Xcc306ΔpthA4</th>
<th>Xcc306ΔpthA4::pthAw vs. Xcc306HrcC-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cit.3027.1.S1_s_at</td>
<td>SWEET1</td>
<td>11.37</td>
<td>6.78</td>
<td></td>
</tr>
<tr>
<td>Cit.5370.1.S1_s_at</td>
<td>Plant invertase/pectin methylesterase inhibitor</td>
<td>9.65</td>
<td>9.26</td>
<td></td>
</tr>
<tr>
<td>Cit.37210.1.S1_at</td>
<td>LOB (related to UPA20)</td>
<td>9.04</td>
<td>15.43</td>
<td></td>
</tr>
<tr>
<td>Cit.18912.1.S1_x_at</td>
<td>germin</td>
<td>7.47</td>
<td>37.11</td>
<td></td>
</tr>
<tr>
<td>Cit.35190.1.S1_at</td>
<td>LOB (same gene as Cit.37210.1.S1_at)</td>
<td>7.08</td>
<td>9.34</td>
<td></td>
</tr>
<tr>
<td>Cit.8649.1.S1_x_at</td>
<td>germin</td>
<td>6.87</td>
<td>37.78</td>
<td></td>
</tr>
<tr>
<td>Cit.15355.1.S1_at</td>
<td>GA20 oxidase. Photosynthesis</td>
<td>6.79</td>
<td>12.50</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3 - Homologs of CsLOB1 in citrus function as disease susceptibility genes in citrus canker

Abstract

The lateral organ boundary domain (LBD) genes encode a group of plant-specific proteins that function as transcription factors in regulating plant growth and development. *Citrus sinensis lateral organ boundary 1* (CsLOB1) is a member of the LBD family and functions as a disease susceptibility (S) gene in citrus bacterial canker (CBC). There are 34 LBD members in *Citrus sinensis*. We assessed the potential for additional members of the LBD genes in citrus to function as surrogates for CsLOB1 in CBC and compared host gene expression upon induction of different LBD genes. By using designed TAL effector (dTALe) genes, another two members, CsLOB2 and CsLOB3, were revealed to be capable of functioning similarly to CsLOB1 in citrus. Through RNA-Seq and qRT-PCR analysis, a set of cell wall metabolic genes were demonstrated to be the potential secondary targets of CsLOB1 in promoting CBC disease.

Introduction

The lateral organ boundary domain (LBD) genes encode a group of plant-specific proteins that function as transcription factors in regulating plant growth and development (Husbands et al., 2007; Majer et al., 2011). All members contain a domain referred as the lateral organ boundary (LOB) domain. The LOB domain contains a cysteine rich motif (CX2CX6CX3C) that has been shown, in some cases, to mediate DNA binding. The proteins also contain a conserved glycine residue and a coiled-coil leucine zipper-like motif (LX6LX3LX6L). *C. sinensis lateral organ boundary 1* (CsLOB1) is a member of the LBD genes and functions as a disease susceptibility (S) gene in CBC (Hu et al., 2014). CBC is an
economically important disease worldwide on most citrus varieties. The typical symptoms are erumpent water-soaked lesions with yellow halos on leaves, stems and fruits. Defoliation and premature fruit drop may occur if disease is severe (Gottwald et al., 2002). The causal pathogens are various species from genus of *Xanthomonas*. Five groups (group A, group A*, group Aw, group B, and group C) of pathovars have been identified, in which group A, A* and Aw belong to the species of *Xanthomonas citri* ssp. *citri* (Xcc) originated from Asia, while group B and C are in the species of *X. fuscans* ssp. *aurantifolii* (Xau) (Al-Saadi et al., 2007; Sun et al., 2004; Verniere et al., 1998). Pustule formation, regardless of the strain, requires *CsLOB1* expression, which is also associated with increases in bacterial populations at the site of infection (Hu et al., 2014; Pereira et al., 2014).

How *CsLOB1* expression facilitates pustule formation and whether *CsLOB1* related genes can function in place of *CsLOB1* are unknown. Forty-three LBD genes have been identified in *Arabidopsis* (Shuai et al., 2002). The founding member of the LBD family in *Arabidopsis*, *AtLOB*, is expressed at the base of lateral organs and involved in leaf development. *AtLOB* functions as a transcription activator and interacts with members of the basic helix–loop–helix (bHLH) family of transcription factors (Husbands et al., 2007; Shuai et al., 2002). Other LBD proteins were demonstrated to regulate anthocyanin and nitrogen metabolism and to respond to phytohormones and environmental stimuli during the process of regulating organ boundary formation (Gendron et al., 2012; Majer et al., 2011). A LBD gene from apple (*Malus domestica* B.), *MdLBD11*, has a similar function as the *Arabidopsis* gene *AtAS2/AtLBD12* for both playing roles in leaf development and flowering (Wang et al., 2013). Expression of *Arabidopsis* gene *LBD20* was induced upon the infection of the pathogen *Fusarium oxysporum* and interference of *LBD20* expression resulted in increased resistance to the pathogen (Thatcher et al., 2012).
CsLOB1 induction during CBC is mediated by one of the six major TAL effectors from strains of Xcc or Xau (Hu et al., 2014; Pereira et al., 2014). PthA was the first member of TAL effector family demonstrated to function in pathogenicity and shown to be required for pustule formation (Swarup et al., 1992). Additional pthA homologous genes were identified from other strains of Xanthomonas and include pthA4, pthAw, pthA*, pthB and pthC (Al-Saadi et al., 2007; Jalan et al., 2013; Sun et al., 2004; Verniere et al., 1998). All of the major TAL effectors target CsLOB1 (Hu et al., 2014; Pereira et al., 2014). TAL effectors are members of type III secretion (T3S) effectors primarily identified in Xanthomonas species and Ralstonia solanacearum (De Feyter et al., 1993; Heuer et al., 2007; Hopkins et al., 1992; Mak et al., 2013). TAL effectors, upon entry into host cells, move to the cell nuclei and bind to the specific promoter regions designated here as effector binding elements (EBEs) of host genes that are targeted for expression (Boch et al., 2014; White et al., 2009b). The cognate EBE is determined by the TAL effector central repetitive region. The amino acid sequence of the repeats is largely conserved with the exception of residues 12 and 13, which are polymorphous and referred as repeat-variable diresidue (RVD) (Boch et al., 2009; Boch et al., 2014; Moscou et al., 2009).

Artificially designed TAL effector (dTALe) genes can be produced by replacing the repetitive region with coding segments that target new binding sites of interest (Li et al., 2012b; Morbitzer et al., 2010; Streubel et al., 2013). The targeting of novel promoter elements by dTALes has been proven useful for discriminating between S genes and phenotypically silent or off-target genes that also contain native EBEs (Cernadas et al., 2014; Cohn et al., 2014; Hu et al., 2014). For example, PthA4 and PthAw both induce CsLOB1 and a second gene CsSWEET1 (Hu et al., 2014). The construction of dTALes that targeted either gene revealed that only the induction of CsLOB1 contributed to pustule formation and an enhanced population level of the pathogen (Hu et al., 2014; Pereira et al., 2014). Furthermore, dTALes could facilitate the identification of S gene surrogates, especially for the paralogs of the identified S
genes. For example, TAL effectors of field strains of *X. oryzae pv. oryzae* are known to target any one of three *OsSWEET* genes (*OsSWEET11, OsSWEET13, OsSWEET14*) encoding closely related members of clade III of sucrose transporters in rice (Antony et al., 2010a; Streubel et al., 2013; Yang et al., 2006; Zhou et al., 2015). The use of dTALes allowed targeting the additional 19 *OsSWEET* genes that are paralogous to the identified S genes of *OsSWEET*. However, only two extra members (OsSWEET12 and OsSWEET15) from clade III could induce the typical disease symptom of water soaking lesions, revealing that only members of clade III can function as S genes (Streubel et al., 2013). Here, we assessed the potential for additional members of the *LBD* genes in citrus to function as surrogates for *CsLOB1* in CBC and compared host gene expression upon induction of different *LBD* genes.

**Methods and materials**

The methods for bacterial inoculation, *Xanthomonas* competent cell preparation and transformation, dTALe construction, bacterial population assay, RNA extraction and qRT-PCR assay should be referred to the methods in Chapter 2. All the bacterial strains and plasmids are shown in Appendix A-1.

**Phylogenetic analysis of *C. sinensis* LBD proteins**

LBD protein sequences were downloaded from the Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/family.php?sp=Csi&fam=LBD). Amino acid sequences were aligned using the SeaView version 4 with the default parameters. The unrooted phylogenetic tree was computed using setting of PhyML-3.1 with the LG amino acid substitution model and the non-parametric bootstrap support test. SeaView 4.1 was used for displaying and editing phylogenetic trees (Gouy et al., 2009).
**Phylogenetic analysis of LBD proteins of CsLOB1-4 and the closely related LBD members in A. thaliana**

Sequences were aligned with the online ClustalW server (http://www.ch.embnet.org/software/ClustalW.html) using the default values. MEGA6.0 was used for generating a tree on the basis of ClustalW output. Phylogenetic calculations were based on the maximum likelihood method, and Bootstrap analysis was used to evaluate the reliability of the nodes of the phylogenetic trees.

**RNA-Seq analysis**

Samples for RAN-Seq analysis were grapefruit leaves inoculated with Xcc306WT, Xcc306ΔpthA4 and Xcc306ΔpthA4::dCsLOB2 which were taken at 5 days post inoculation (dpi). RNA was extracted using TRIzol reagent (Life Technology) and purified by a 7.5M LiCl solution according to the manufacturer’s protocol (Life Technology). For each strain, three replications were performed. Libraries were constructed using the TruSeq™ RNA and DNA Sample Prep Kits (Illumina). Nine samples were pooled into one lane for illumine High Output 100 cycle single end (HO-SR100) sequencing.

Data was analyzed using software RobiNA from MapMan. For trimming the adapters, Trimmomatic with the default settings was used (Bolger et al., 2014). The trimmed fastq files were aligned with the reference genome sequence and annotation file of Csinensis_154 from Phytozome v10 using BOWTIE with the default settings except the maximal number of mismatches was changed into 2 (Langmead et al., 2009). When the mapping was finished, the counts table was computed by the RobiNA program automatically. For differential expression analysis, edgeR was used (Robinson et al., 2010). False discovery rate (FDR) was calculated from P values according to Benjamini and Hochberg’s algorithm (Benjamini et al., 1995). A corrected P value of cutoff 0.05 was applied.
Gene annotation and enrichment were analyzed using online software Mercator and downloaded software MapMan. For the MapMan, a modified mapping file of Csinensis_154 was used for analyzing the potential pathways regulated by LBD members.

Results

Phylogeny of citrus LBD gene family

The three citrus genes most closely related to CsLOB1 based on the C. sinensis proteome are orange1.1g045080m, orange1.1g047491m and orange1.1g036782m. Moreover, thirty four members in C. sinensis LBD Family were revealed in the Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/family.php?sp=Csi&fam=LBD). None has been characterized genetically with the exception of the function of CsLOB1 (orange1.1g026556m) in CBC (Hu et al., 2014). The constructed phylogenetic tree was divided into eight clades. The clade with CsLOB1 was chosen as clade I and harbors the four additional members orange1.1g047491m, orange1.1g045080m, orange1.1g047530m, and orange1.1g047804m (Figure 3-1). Two of the four LBD proteins from clade 1 (orange1.1g045080m and orange1.1g047491m) and one (orange1.1g036782m) from the next clade (arbitrarily named clade II) were chosen for further analysis and dTALe targeting (Figure 3-1, red arrows). Hereafter, the three LBD genes (orange1.1g045080m, orange1.1g047491m and orange1.1g036782m) were designated as CsLOB2, CsLOB3 and CsLOB4, respectively.

Pustule symptoms are restored by dTALes targeting CsLOB2 and CsLOB3 but not CsLOB4

The promoter regions of CsLOB2, CsLOB3 and CsLOB4 were scanned for potential EBEs using TAL Effector Nucleotide Targeter 2.0 (https://tale-nt.cac.cornell.edu/node/add/talef-off). Sites closest to TATA box were selected (Figure 3-2A). The dTALes were constructed using the backbone of pthAv and optimized RVDs for the selected EBE sequences (Figure 3-2B) (Li et al., 2013). The genes were
designated as dCsLOB2, dCsLOB3, and dCsLOB4 (Figure 3-2A and B). The functional abilities of the dTALEs were tested by qRT-PCR of mRNAs from grapefruit leaves after inoculation with pthA4 mutant strain 306ΔpthA4, and the same strain complemented by introduction of the individual dTALE genes (dCsLOB2, dCsLOB3, and dCsLOB4). The results showed that, compared to 306ΔpthA4, the complemented strains induced significant higher fold changes for the respective targets (Figure 3-3).

The contribution of respective strains harboring dTALEs for the various LBD genes to disease symptom development was tested on grapefruit leaves. At 15 days post inoculation (dpi), the sites with inoculum of either Xcc306ΔpthA4::dCsLOB2 or Xcc306ΔpthA4::dCsLOB3 showed the pustule formation, and the sites with either Xcc306ΔpthA4 or Xcc306ΔpthA4::dCsLOB4 only showed water soaking (Figure 3-4).

**The dTALE dCsLOB2 promoted an increase in bacterial populations on citrus leaves**

To determine if induction of an alternative LBD member is also associated with the bacterial population increases on citrus leaves, bacterial population assays were conducted with strains of Xcc306WT (with pthA4), 306ΔpthA4 and Xcc306ΔpthA4::dCsLOB2. Indeed, Xcc306ΔpthA4::dCsLOB2 achieved higher populations than that of 306ΔpthA4 and comparable to the population of strain Xcc306WT after 3 days on host plants (Figure 3-5). To exclude the possibilities that the pustule formation or population promotion conferred by CsLOB2 or CsLOB3 might be associated with the co-incidental expression of CsLOB1, the induction of CsLOB1 in the presence of dCsLOB2 or dCsLOB3 was assayed. The result showed no significant induction of CsLOB1 by Xcc306ΔpthA4::dCsLOB2 or Xcc306ΔpthA4::dCsLOB3 relative to Xcc306ΔpthA4 (Figure 3-6).
**S gene expression does not contribute to enhanced virulence of a non-host pathogen**

*X. campestris pv. vesicatora* (Xcv) Xcv8510, a pathogen of pepper, was used as a non-host strain to determine if a TAL effector gene could enhance the virulence of Xcv on a non-host. Xcv8510 normally induces light water-soaking on citrus (Figure 3-6A). CsLOB2 was highly induced by XcV8510::dCsLOB2 when inoculated on grapefruit leaves compared to the control Xcv8510::PHM (empty vector) as determined by qRT-PCR (Figure 3-6B). Pustule-like symptoms were observed at the inoculation sites with XcV8510::dCsLOB2 (Figures 3-6A). However, Xcv8510, which grows to relatively low population levels compared to Xcc306WT, did not grow to higher populations with the help of the dCsLOB2 in comparison to the strain without the gene (Fig. 3-6C).

**Comparison of genes induced in association with CsLOB1 or CsLOB2 expression**

CsLOB1 and CsLOB2 might accomplish CBC development by regulating common downstream genes and pathways. RNA-Seq analysis was used to investigate the transcriptome profile of infected tissues with strains of the pathogen that induce either CsLOB1 or CsLOB2. Grapefruit leaves were inoculated with Xcc306ΔpthA4 (negative control), Xcc306WT, which induces CsLOB1 via PthA4, or Xcc306ΔpthA4::dCsLOB2 with the dTALe that targets CsLOB2. Approximately 22 million 100-nt single-end reads were obtained from cDNA libraries of three replicates for each of the three treatments. Approximately 57% of the reads from each sample were mapped to the *C. sinensis* genome sequences (Csinensis_154 from Phytozome v10). Pairwise comparisons were made between Xcc306WT and Xcc306ΔpthA4 and between Xcc306ΔpthA4::dCsLOB2 and Xcc306ΔpthA4 to compute log2 (fold change) of individual gene expression. The comparison of Xcc306WT and Xcc306ΔpthA4 generated the downstream genes regulated in association with CsLOB1 expression, and the comparison of Xcc306ΔpthA4::dCsLOB2 and Xcc306ΔpthA4 produced the gene expression profile triggered by CsLOB2 expression. The analysis resulted in 4960 genes whose differential expression levels were
significant at the level of FDR < 0.05 in both of the two comparisons - Xcc306ΔpthA4::dCsLOB2 vs. Xcc306ΔpthA4 and Xcc306WT vs. Xcc306ΔpthA4. Among these 4960 genes, expression in 2744 was down-regulated, while 2188 were up-regulated (Figure 3-7).

Both Mercator and MapMan data analysis software were used to predict pathways that are associated with CsLOB1 and CsLOB2 expression. For Mercator analysis, a threshold of log2 (fold change) of larger than 1 of the 4960 genes in comparisons of Xcc306WT vs. Xcc306ΔpthA4 and Xcc306ΔpthA4::dCsLOB2 vs. Xcc306ΔpthA4 was set, and 1429 transcripts were apportioned. The Mercator analysis of these 1429 transcripts indicated that cell wall related genes constituted 7.46% of the genes (Figure 3-9).

The metabolic overview software MapMan maps transcripts as single annotated gene in a specific pathway with each colored square denoting the direction of expression change from samples. Many of the 4960 genes that were significantly regulated in the presence of both CsLOB1 and CsLOB2 were categorized into cell wall, lipid, second metabolism and light reaction pathways (Figure 3-10A and B). Among the up-regulated (blue) genes, most were classified into cell wall related genes, while genes in the other categories were mostly down regulated (Figure 3-10A and B).

**Validation of the induction of cell wall related genes in association with CsLOB1-4**

Expression assays were conducted using qRT-PCR to validate the induction of selected cell wall related genes in the presence of the various _LBD_ gene expressions. Representative genes from the gene lists of cell metabolic pathway acquired from the above Mapman analysis were selected with high fold changes for validation. Among the selected genes, three are cell wall degradation genes including a beta-1,4-glucanase gene (orange1.1g010632m), a cellulase gene (orange1.1g014426m) and a pectate lyase gene (orange1.1g015623m), two are cell wall modification genes including a beta-expansin 3 gene
(orange1.1g023962m) and an alpha-expansin 9 gene (orange1.1g024916m), one cellulose synthase-like gene (orange1.1g006104m) and one pectinesterase gene (orange1.1g015794m). A strictosidine synthase gene (orange1.1g046107m) and a reductase gene (orange1.1g040244m) were also chosen as representatives of dCsLOB2-specific genes according to the results from RNA-Seq analysis to test the CsLOB2 specificity.

Validation of the results from the RNA-Seq analysis were conducted by qRT-PCR of independent RNA samples from grapefruit leaves treated with Xcc306WT, Xcc306ΔpthA4, Xcc306ΔpthA4::dCsLOB1 or Xcc306ΔpthA4::dCsLOB2. The results were consistent with the RNA-Seq analysis, and all the selected cell wall relate genes were up-regulated in response to inoculations by Xcc306WT, Xcc306ΔpthA4::dCsLOB1 or Xcc306ΔpthA4::dCsLOB2 relative to Xcc306ΔpthA4. The two CsLOB2-specific transcripts were only induced in the presence of Xcc306ΔpthA4::dCsLOB2 relative to Xcc306ΔpthA4 (Figure 3-11A).

The induction of LBD associated cell wall related genes was also assayed by qRT-PCR in association with expression of CsLOB2 during challenged by a Xanthomonas species with a different genetic background from Xcc, namely Xcv8510. Therefore, the qRT-PCR was conducted with Xcv8510::PHM or Xcv8510::dCsLOB2 infected tissues to test the same set of genes as above. The results indicated that dCsLOB2 expression was associated with the induction of the three cell wall degradation genes (a beta-1,4-glucanase gene, a cellulase gene and a pectate lyase gene) and the two cell wall modification genes (a beta-expansin 3 gene and an alpha-expansin 9 gene), while the cellulose synthase-like gene and pectinesterase gene were not induced by Xcv8510::dCsLOB2 relative to Xcv8510::PHM. The non-host Xcv8510 induced a smaller set of cell wall related genes in comparison to Xcc306 (Figure 3-11A and B).
The cellulose synthase-like gene and the pectinesterase gene were removed from the selected set of cell wall related genes due to their poor response to Xcv8510::dCsLOB2 inoculation. The expression profiles of the remaining subset of the selected cell wall related genes were assayed by qRT-PCR in the presence the additional two LBD genes, namely CsLOB3 and CsLOB4, which were not included in the original RNA-Seq assays. The results indicated that CsLOB3 and CsLOB4 expression was also associated with cell wall related genes expression, and all five were induced by Xcc306ΔpthA4, carrying either dCsLOB3 or dCsLOB4 at the significance levels of $P < 0.05$. However, the induction levels associated with CsLOB4 expression were, at maximum, one-fifth the levels in association with CsLOB3 expression (Figure 3-11C).

**Discussion**

The results indicate that more than one member of LBD genes can serve as an S genes in CBC. Here, two additional LBD family members, CsLOB2 and CsLOB3, were capable of directing pustule formation when strains of Xcc containing LBD-specific dTALe genes were inoculated on citrus leaves. TAL effector-mediated induction of CsLOB4 failed to support pustule formation, indicating that at least one member was incapable of supporting pustule formation. CsLOB2 and CsLOB3 are both members of the same clade as CsLOB1, while CsLOB4 lies in a close but distinct clade of LBD family members. At the same time, it is premature to conclude whether pustule formation is limited to clade I members, since only one member outside of clade I was tested. The induction of CsLOB1, CsLOB2 and CsLOB3 by the respective TAL effectors facilitated symptom development and, at least in the case of CsLOB1 and CsLOB2, also promoted pathogen population levels in leaves. Like the situation of the SWEET genes in bacterial blight of rice in which five SWEET genes from one clade could serve as S genes, the results may reflect a common biochemical function of the genes within a specific clade. However, differently from bacterial blight of rice where three SWEET genes are targeted by field strains, only one member of
clade I has been shown to function as an S gene for CBC in the field (Antony et al., 2010a; Hu et al., 2014; Pereira et al., 2014; Yang et al., 2006; Zhou et al., 2015). The reason might be attributed to breeding histories for rice and citrus. Genetic breeding for rice can be traced back to 10,000 years ago (Asano et al., 2011). The repeated deployment of genes for resistance in rice to bacterial blight for specific TAL effector genes in X. oryzae pv. oryzae, and the long breeding history undoubtedly has had an effect on the pathogen population gene content, and increased chance for diversification in S gene targets (Garris et al., 2005; Shinada et al., 2014). The documented breeding history of citrus is for a much shorter time, perhaps 200 years, and, as a tree species, breeding cycles are considerably longer (Khan, 2007). Therefore, the genetic complexity of the pathogen/plant interactions may be much lower in citrus than that in rice. The relatively simpler gene pool puts less selection force on S gene targeting for CBC in nature. The results also indicate that the CBC causal strains are broadly at risk, at least in the short term, to recessive resistance strategies involving polymorphisms in the CsLOB1 promoter.

Though certain LBD members have been characterized, little is known about the biological functions of LBD members in citrus. The constructed phylogenetic tree (Figure 3-12A) shows that CsLOB1, CsLOB2 and CsLOB3 are most closely related to AtLBD1 and AtLBD11, while CsLOB4 is more closely related to AtLBD12. The protein sequence alignment reveals the consensus LOB domain that contains the three conserved motifs, a cysteine rich domain CX2CX6CX3C, a conserved glycine residue, and a coiled-coil leucine zipper-like motif LX6LX3LX6L (Figure 3-12B). The AtLBD12 and CsLOB4 have much shorter N terminals, which might account for the dysfunction of CsLOB4 as an S gene in CBC. However, the reports on the function of AtLBD1 or AtLBD11 are few (Matsui et al., 2008). AtLBD12 mutants were reported to have reduced leave size, apical dominance, and epinastic leaves, and the mutant plants had increased sterility (Nakazawa et al., 2003). Therefore, the prediction of the biological functions of CsLOB1, CsLOB2 and CsLOB3 are premature based on the homologies.
Nonetheless, the results indicate that \textit{CsLOB1}, \textit{CsLOB2} and \textit{CsLOB3} regulated a common set of as yet unidentified downstream genes that ultimately trigger pustule development. Possibly, the pustule-associated \textit{LBD} genes bind common promoter elements. However, the fact that several genes were found to be induced in the presence of \textit{CsLOB2} and not \textit{CsLOB1} indicates overlapping but not entirely the same set of downstream events for the transcription factors.

\textit{Citrus LBD} genes, as a family of transcription factor genes, potentially function in regulating downstream pathways for plant normal development. The RNA-Seq and qRT-PCR results here revealed that a subset of cell wall related genes, especially those genes involving in cell wall degradation and cell wall modification, were induced when the pustule-associated \textit{LBD} genes were present. While by \textit{CsLOB4}, one member incapable of forming pustules, much lower levels of expression for the cell wall related genes were observed, which indicates that these cell wall related genes maybe functionally important for pustule formation and symptom development. The plant cell wall acts as a physical barrier in interactions with plant pathogens. On one side, plant cell walls inhibit the pathogen infection. On the other side, pathogens employ various strategies to manipulate their host plant cell wall metabolism to favor their own infection (Bellincampi et al., 2014). In tomato, several genes involving pathways of fruit ripening function as susceptibility factors when infected by \textit{Botrytis cinerea}, which are Cel1 and Cel2 (endo-b-1,4-glucanase), LePG (polygalacturonase) and LeExp1 (expansin). Down-regulation of these genes attenuated the susceptibility of tomato fruits to pathogen \textit{B. cinerea} (Cantu et al., 2009; Flors et al., 2007). The qRT-PCR results associated with the dTALes d\textit{CsLOB1}, d\textit{CsLOB2} and d\textit{CsLOB3} reflect the possible role of a set of cell wall metabolic genes as susceptibility factors. \textit{CsLOB1}, \textit{CsLOB2} and \textit{CsLOB3} as transcription factors might function in different tissues in regulating normal fruit ripening by inducing the cell wall associated fruit ripening genes. Targeting of individual downstream citrus genes may allow identification of specific critical genes using the dTALe strategy.
The clade I LBD genes of citrus join a limited club of TAL effector dependent S genes that have been shown to contribute major phenotypic features to the respective disease processes. Three other examples have been characterized. The only one for bacterial spot of pepper is *upa20*, encoding a bHLH family transcription factor, the S gene targeted by TAL effector AvrBs3 from *X. campestris* pv.*vesicatoria*. UPA20 was revealed to play possible roles in cell enlargement in pepper bacterial leaf spot disease (Kay et al., 2007). *OsSULTR3;6*, a putative sulfate transporter gene is targeted by Tal2g of *X. oryzae* pv.*oryzicola* in rice bacterial leaf streak disease, where the gene facilitates lesion expansion and bacterial exudation (Cernadas et al., 2014). The largest group are the SWEET genes of rice and cassava (Antony et al., 2010a; Cohn et al., 2014; Streubel et al., 2013; Yang et al., 2006; Zhou et al., 2015). These SWEET proteins have been demonstrated to function in transporting sucrose or glucose during pathogenesis (Chen et al., 2010; Chen et al., 2012; Zhou et al., 2015). The range of genes that have been identified to date indicates that a variety of host genes can serve as S genes. At the same time, little is known regarding the function of TAL effector-dependent S genes from a physiological perspective. Characterization of additional pathosystems that involve TAL effector-mediated virulence may ultimately reveal common pathways for enhancing host susceptibility.
Figure 3-1 Phylogenetic tree of *C. sinensis* LBD genes.

Values with cutoff of 70 for the bootstrap supports (500 replicates) are shown on the shoulder of branches. The scale bar indicates substitutions/site and vertical lines are used to represent different clades of *C. sinensis* LBD proteins.
**Figure 3-2 DTALe design and construction for CsLOB2, CsLOB3 and CsLOB4.**

A. Partial promoter regions of three targeted LBD genes in *C. sinensis*. The yellow highlights the EBEs that the dTALes target, and the red letters indicate the predicted TATA boxes and start codons for each gene. B. The repeat variable di-residues (RVDs) of three dTALes and the corresponding EBEs in the promoters of CsLOB2, CsLOB3 and CsLOB4. The TAL code is on the basis of Grau et al (Figure 1-2) (Grau et al., 2013).
Figure 3-3 The targeted LBD genes are induced by the respective dTALes.

I: Xcc306ΔpthA4, II: Xcc306ΔpthA4::dCsLOB2, III: Xcc306ΔpthA4::dCsLOB3 and IV: Xcc306ΔpthA4::dCsLOB4. QRT-PCR results of mRNA extracted from grapefruit leaves inoculated with the individual Xcc strains as indicated. Error bars represent the standard deviations for three independent experimental replicates. Samples were taken at 5dpi. Asterisks (*) denote significant differences in the fold changes between the treatments and the negative control Xcc306ΔpthA4 at $P < 0.05$ using t-test.
Figure 3-4 Disease symptoms induced by dTALes targeting the three *CsLOB1*-related genes.

A. Pictures were taken using canon digital SLR camera. B. Imaged by dissecting microscope camera (6.4X). 1, Xcc306WT; 2, Xcc306ΔpthA4::dCsLOB2; 3, Xcc306ΔpthA4::dCsLOB3; 4, Xcc306ΔpthA4::dCsLOB4; 5, Xcc306ΔpthA4; 6: healthy leaf.

Photographs were taken at 15 dpi of various strains as shown on the pictures. Inocula were 10^3X dilution of bacterial suspensions of OD_{600}=0.5.
Figure 3-5 DCsLOB2 promoted the increase of bacterial population in host plants.

Bacterial population assay were executed for three strains: Xcc306ΔpthA4, Xcc306WT, and Xcc306ΔpthA4::dCsLOB2 on grapefruit. X-axis shows the days post inoculation. The stars indicate the significant difference (P < 0.01) of two comparison, Xcc306WT vs Xcc306ΔpthA4 and Xcc306ΔpthA4::dCsLOB2 vs Xcc306ΔpthA4. Statistical significance analysis were tested using ANOVA and t-test.
Figure 3-6 *CsLOB1* is not induced by the dTALes targeting *CsLOB2* or *CsLOB3*.

QRT-PCR results of mRNA extracted from grapefruit leaves inoculated with the individual Xcc strains as indicated. Error bars represent the standard errors for three independent experimental replicates. Samples were taken at 5dpi. Different letters indicate significant difference in the fold changes among the treatments at $P < 0.05$ using t-test.
Figure 3-7 S gene expression does not contribute to enhanced virulence of a non-host pathogen.

A. DCsLOB2 induced pustule-like symptom when transformed into Xcv8510. Picture were taken at 30dpi of grapefruit leaves inoculated with Xcv8510::PHM or Xcv8510::dCsLOB2 using either general camera(1-4) or dissecting microscope camera at magnification of 6.4X (5,6). Inocula of OD$_{600}$=0.5 (1,2) or the same inocula diluted into 10$^3$ times(3-6). B. DCsLOB2 induced CsLOB2 expression when transformed into Xcv8510. qRT-PCR of RNA samples of grapefruit leaves inoculated with Xcv8510::PHM and Xcv8510::dCSLOB2 (5dpi). Inocula were bacterial suspension of OD$_{600}$=0.5. Samples were take at 5dpi. Error bars represent the standard errors for three independent experimental replicates. * indicates significant difference between the two treatments (t-test $P < 0.05$). C. Bacterial population assay of two strains: Xcv8510::PHM and Xcv8510::dCSLOB2 on grapefruit. T-test ($P < 0.05$) showed no significant difference between the two treatments.
Figure 3-8 RNA-Seq results of gene expression profile induced by CsLOB1 and/or CsLOB2.

Venn diagrams illustrating the number of genes that were differentially expressed in each comparison (Xcc306WT vs. Xcc306ΔpthA4 and Xcc306ΔpthA4::dCsLOB2 vs. Xcc306ΔpthA4) at the FDR < 0.05 level. The comparisons are represented by green or red circle. Genes that show significant differences in two comparisons are plotted in the overlapping areas. The amount of unchanged genes is given in the lower right corner of the plots.
Figure 3-9 Mercator functional category assignments of common genes that up regulated in association with CsLOB1 and CsLOB2 expression.

Mercator analysis of the genes in grapefruit with expression fold change larger than 1 in two comparisons: Xcc306WT vs. Xcc306Δpth A4 and Xcc306Δpth A4::dCsLOB2 vs. Xcc306Δpth A4 (1429 elements) at 5dpi.
Figure 3-10 Mapman metabolic overview of the genes regulated by LBD family members.

A. Genes expression associated with Xcc306ΔpthA4::dCsLOB2 relative to Xcc306ΔpthA4 (A) and Xcc306WT relative to Xcc306ΔpthA4 (B). Log2 (fold changes) are indicated as a gradient between red (top, down-regulated) and blue (bottom, up-regulated) as shown in the indication bars.
A

Gene relative expression level (2^ΔΔCT)

Gene

beta-1,4-glucanase
Cellulase
Pectate lyase
Beta-expansin 9
Alpha-expansin 9
Cellulose synthase
Pectinesterase
Sporopollenin synthase
Reconstit

B

Gene relative expression level (2^ΔΔCT)

Gene

beta-1,4-glucanase
Cellulase
Pectate lyase
Beta-expansin 9
Alpha-expansin 9
Cellulose synthase
Pectinesterase
Sporopollenin synthase
Reconstit

C

Gene relative expression level (2^ΔΔCT)

Gene

beta-1,4-glucanase
Cellulase
Pectate lyase
Beta-expansin 9
Alpha-expansin 9
Cellulose synthase
Pectinesterase
Sporopollenin synthase
Reconstit

Legend:

- Xc306WT
- 306ΔΔphaA
- 306ΔΔphaA::dCalOB1
- 306ΔΔphaA::dCalOB2
- XcV8510::PHM
- XcV8510::dCalOB2

70
Figure 3-11 QRT-PCR validation of cell wall-related genes induced by dCsLOB1, dCsLOB2, dCsLOB3 or dCsLOB4.

A and C: Grapefruit leaves treated with various Xcc strains as indicated in the figure. Samples were taken at 5dpi. Inocula were bacterial suspension of OD$_{600}$=0.5. B: Grapefruit leaves treated with various Xcv8510 strains as indicated. Samples were taken at 5dpi. Inocula were bacterial suspension of OD$_{600}$=0.5. Error bars represent the standard errors for three independent experimental replicates. Different letter indicates significant difference ($P < 0.05$) using ANOVA analysis and t-test. The phytozone ID for each gene is beta -1,4-glucanase gene (orange1.1g010632m), cellulase gene (orange1.1g014426m), pectate lyase gene (orange1.1g015623m), beta-expansin 3 gene (orange1.1g023962m), alpha-expansin 9 gene (orange1.1g024916m), cellulose synthase-like (orange1.1g006104m), pectinesterase (orange1.1g015794m), strictosidine synthase (orange1.1g046107m), reductase (orange1.1g040244m).
Figure 3-12 LBD proteins of *CsLOB1, CsLOB2, CsLOB3, CsLOB4* and closely related LBD members in *A. thaliana*.

A. A phylogenetic tree of LBD proteins of *CsLOB1, CsLOB2, CsLOB3, CsLOB4* and closely related LBD members in *A. thaliana*. A monophyletic group that contains *CsLOB1, CsLOB2, CsLOB3* is boxed in red. The group that contains CsLOB4 is boxed in blue. Bootstrap values were based on 500 replications. The branch lengths of the tree are proportional to divergence. The 0.1 scale represents 10% change. B. Protein sequence alignment of LBD genes from *Citrus sinensis* (Cs) and *Arabidopsis thaliana* (At). Three conserved motifs, CX2CX6CX3C, glycine residue, and LX6LX3LX6L, are highlighted with red, pink, and blue, respectively. The alignment was conducted using CLUSTALW with the default settings.
**Chapter 4 - Functional analysis of TAL effectors in X. axonopodis pv. glycines**

**Abstract**

Soybean bacterial pustule (SBP), caused by *X. axonopodis pv. glycines*, is a common disease of soybean in regions with high temperatures and humidity. The typical symptoms are the small pale-colored erumpent pustules surrounded by yellow halos. Six TAL effectors with different repeat sequences were characterized for a *X. axonopodis pv. glycines* strain Xag12-2 using Southern hybridization assays and TAL effector gene mutagenesis. One TAL effector TAL2 were shown to have a virulent effect in terms of pustule formation and population growth on soybean plant hosts. Furthermore, through genome transcriptom profiling by RNA-Seq, two candidate plant susceptibility (S) genes of TAL2 were identified. One candidate gene encodes a zinc-finger-homeodomain (HD-ZF) transcription factor, and the other one encodes an aluminum activated malate transporter (ALMT). Candidate genes from families of *LBD* and *bHLH* transcription factors were not detected.

**Introduction**

Soybean bacterial pustule (SBP), caused by *X. axonopodis pv. glycines*, is a common disease of soybean with higher occurrence under high temperatures and humidity (Prathuangwong et al., 1987). The typical symptoms are the small pale-colored erumpent pustules surrounded by yellow halos. The size of the pustule area varies from small specks to large necrotic areas. Severe infections often lead to an early defoliation, which may reduce the soybean yield (Morgan, 1963; Weber et al., 1966). The bacteria enter into the plants through natural openings and wounds and then multiply in the substomatal chambers and the intercellular spaces (Jones et al., 1985). Histological studies of infected tissues revealed both cell enlargement (hypertrophy) and cell multiplication (hyperplasia) were involved in
pustule formation (Jones et al., 1985; Jones et al., 1987). Exopolysaccharide (EPS) seems to play a role in the early infection stage (Jones et al., 1985; Jones et al., 1987). Other bacterial produced chemicals also contribute to pustule formation or SBP development including auxin (Fett et al., 1987), toxin, cellulase, pectinases (Hayward, 1993), sucrose hydrolase (H. S. Kim et al., 2004) and bacteriocins (Fett et al., 1987).

A hemin transport gene (hem) was shown to be a virulence factor by affecting the epiphytic fitness of *X. axonopodis pv. glycines* strains tested by loss of function assay (Prathuangwong et al., 2013). The leaf population size and the disease incidence were reduced in the hem mutant. Further tests demonstrated that Hem transporter proteins might function in extracellular polysaccharide production, biofilm formation, bacterial motility and bacterial attachment to host plant surface (Prathuangwong et al., 2013). *X. axonopodis pv. glycines* harbors the conserved gene called *rpfF*, which encodes an enzyme for the production of an extracellular compound known as diffusible factor (Thowthampitak et al., 2008). Mutation of *rpfF* resulted into a decreased virulence on soybean, reduced productions of extracellular polysaccharide and enzymes such as carboxymethylcellulase, protease, endo-β-1,4-mannanase, and pectate lyase (Thowthampitak et al., 2008). The transcription regulator XagR is also essential for full virulence, contributing to cell egress by regulating expression of a variety of genes including *pip* that encodes proline iminopeptidase, functioning as a virulence factor in a range of *Xanthomonas* strains and adhesion gene *yapH* (Chatnaparat et al., 2012; Das et al., 2009; Zhang et al., 2007).

TAL effectors may have important contributions to *X. axonopodis pv. glycines* pathogenicity. The TAL effector gene profiles of 155 strains from Korea were investigated. Six patterns were revealed by DNA hybridization, and 3 to 7 TAL effector genes were detected in individual strains. All but one strain were shown to contain TAL effector-related 3.2 kb BamHI fragment (Park et al., 2008). Virulence assays of the strains with different TAL effector gene complements provided additional correlative...
evidence that one or more TAL effector genes might contribute to virulence (Park et al., 2008). However, the specific contributions of each TAL effector to bacterial virulence were not determined (Park et al., 2008). Subsequently, the TAL gene \textit{avrXg1} was isolated from \textit{X. axonopodis pv. glycines} wild type race 3 strain KU-P-SW005 and demonstrated to function as an Avr gene in one race of the pathogen and a virulence factor in another race (Athinuwat et al., 2009). Here, we examined the TAL effector gene content of \textit{X. axonopodis pv. glycines} strain Xag12-2 in preparation to identify candidate host genes of soybean that serve as susceptibility (S) genes. Ultimately, we will compare host gene induction in various diseases that are caused by members of the species \textit{X. axonopodis} and closely related species.

\textbf{Methods and materials}

\textit{Plant material, bacterial strains, and plasmids}

Seeds of the soybean cultivar Spencer were kindly provided by Dr. Steve Lindow and Stella Kantartzi. Seeds were germinated on a wet filter paper at 28°C under dark condition for 2 days, and transferred into pots filled with Bacto Premium Potting Soil (Hummert International). Growth conditions were 25°C, 80% humidity, 16 hour day length and 8 hour dark. All \textit{Xanthomonas} strains were grown at 28°C. The culture temperature for \textit{E.coli} was 37°C. All the bacterial strains and plasmids are shown in Appendix A-1.

\textit{Genomic DNA extraction from Xag12-2 strains}

A loop of bacteria was collected into 1ml sterile distilled water and was washed once with sterile water and collected by centrifugation. The genomic DNA was prepared using the protocol of Wilson’s (Wilson, 2001).
**TAL effector cloning**

The genomic DNA was digested by *Bam*HI, and used to replace the *Bam*HI fragment of the plasmid pzw-pthAw (Appendix A-1). The DNA sequencing of the repeats was accomplished by introducing primers sites into the repetitive sequence through Tn5 mutagenesis (EZ-Tn5™ <KAN-2> Insertion Kit, Epicentre).

**Xag12-2 TAL effector gene mutagenesis**

TAL effector gene mutations were generated by homologous recombination of a defective TAL effector gene carrying a Tn5 insertion, which confers resistance to the antibiotic kanamycin. Xag12-2 wild type strain was transformed with a non-replicable plasmid containing the mutated TAL effector gene. Colonies were selected for resistance to kanamycin, and subjected to Southern blot hybridization analysis. The SphI fragment of the TAL effector gene *avrXa7* was used as probes and labeled with a commercial kit according to the manufacturer’s protocol (DIG-High Prime DNA Labeling and Detection Starter Kit I, Roche).

**Plant inoculation**

Single colonies were chosen for each strain and cultured on TSA plate at 28°C for 2 days under individual antibiotic selections. Suspensions were made from plated bacteria to OD<sub>600</sub>=0.2, and further diluted into 10<sup>3</sup> times using autoclaved distilled water. For the disease phenotype and bacterial population assay, 1ml syringe without a needle was used to inject the bacteria into plant leaves. For the virulence scoring, pustule numbers were counted within a circular area of 0.2 cm<sup>2</sup> at each inoculation sites. At least ten sites were scored for each inoculated strain.
**Bacteria population assays**

Eight leaf discs (area=0.28cm²) from leaf tissues inoculated with the respective strains were taken using five ml diameter corker borer. The samples were ground in 10ml autoclaved distilled water. The grounded suspensions were subjected to ten-fold serial dilutions. 50ul aliquots of bacterial suspensions of three serial concentrations were plated on the TSA plates with appropriate antibiotics. The number of colonies on each plate was scored after incubation at 28°C for two days,

**RNA-Seq assays**

Leaves were inoculated with bacterial suspensions (OD₆₀₀=0.2) of Xag12-2 wild type or the Xag12-2 *Tal2* mutant with and without 100 µM cycloheximide (CHX), and the respective tissues were collected for RNA extraction at 24 hours post inoculation (hpi). The 24 hpi time point was chosen to allow time for the induction of the bacterial type III secretion system and, also at this time, the leaves are still alive and attached to the plants. If taken at a later time, the leaves may drop from the plants. Six pieces of leaves per replicate and three replicates were used for each treatment. RNA was extracted using TRIzol® Reagent (Life Technologies). The RNA samples were sent to Genome Sequencing Core in the University of Kansas for library construction and High Output 100 cycle single end (HO- SR100) sequencing. Genome assembly and gene sequence files of Gmax 189 from phytozome were used as reference sequences. Prinseq (Schmieder et al., 2011), Tophat2 (D. Kim et al., 2013), Cuffmerge (Trapnell et al., 2010) and Cuffdiff2 (Trapnell et al., 2013) were used to acquire the fragments per kilobase (FPK) of exon model per million mapped fragments (FPKM) values for each sample. The p-values and q-values were generated by Cuffdiff2 (Trapnell et al., 2013).
Candidate gene promoter retrieval and analysis

The DNA sequences of 1kb upstream from the initiation codon ATG of the two candidate S genes were retrieved from phytozome according to the transcript IDs and were scanned for the putative EBEs targeted by TAL2 using TALgetter web-server (Grau et al., 2013).

Results

The TAL effector profile of Xag12-2

The profile of TAL effectors was investigated in order to functionally analyze the TAL effector genes in Xag12-2. Southern blot analysis revealed six candidate TAL effector genes based on fragments that hybridized with the probe. The sizes of Sphi digestion fragments ranged from 2kb to 3kb (Figure 4-1A). The DNA sequencing of the repeat regions of the six TAL effector genes showed that the numbers of repeats in the repetitive regions of the respective TAL effectors ranged from 15 to 23 (Figure 4-1B). Tal2 gene is similar to the previously identified TAL effector gene avrXg1 from X. axonopodis pv. glycines wild type race 3 strain KU-P-SW005, with only three different RVDs in the middle of the repetitive region (Figure 4-1C).

The virulence of Xag12-2 was altered by loss of TAL2

Strains were selected with a mutation in each of the candidate TAL effector gene on the basis of fragment polymorphisms after Southern blot analysis for functional analysis of virulence of each TAL effector in Xag12-2, and the virulence of the mutated strains were tested in soybean leaves. The results showed that only strains carrying Tal2 mutations showed reduced virulence on the basis of less pustule formation (Figure 4-2A, B and C) and decreased population levels (Figure 4-2D).
**Candidate targeted S genes of TAL2**

Gene expression profiles were investigated using RNA-Seq assay to uncover the potential S genes targeted by TAL2. Gene expression levels were measured for plant leaf tissues inoculated with either Xag12-2 (wild type) or Xag12-2 - S12 (*Tal2* mutant). Expression profiles were conducted with and without cycloheximide (CHX). CHX can inhibit protein biosynthesis in eukaryotic organisms (Schneider-Poetsch, et al., 2010). Use of CHX is based on the idea that only preformed transcription factors, including TAL effectors, which are synthesized in bacterial cells and secreted into plant cell, can promote gene expression due to the presence of CHX (Boch et al., 2014). Genes with $q$ values (false discovery rate) smaller or equal to 0.05 were chosen for further analysis. Genes that satisfied the following conditions were considered as candidate S genes: (1) Values of log2 (fold change of FPKM$_{WT}$/FPKM$_{S12}$) were greater or equal to 1; and (2) FPKM$_{WT}$ and log2 (fold change of FPKM$_{WT}$/FPKM$_{S12}$) were not significantly reduced in the presence of CHX. Two candidate S genes were selected based on the filter requirements. The first one (Glyma20g04880) was predicted to encode a member of the zinc-finger-homeodomain (ZF-HD) transcription factor family and the second one (Glyma05g35190) was annotated as a gene encoding a member of the aluminum activated malate transporter (ALMT) family (Table 4-1). TAL2-dependent expression of the two S genes was also measured by qRT-PCR (Figure 4-3). The result showed that the expression of the two S genes under condition of either with or without CHX, were significantly different in plant tissues inoculated with the wild type strain relative to the tissues inoculated with the *Tal2* mutant (S12) ($P < 0.05$) (Figure 4-3).

The promoters of the two candidate S genes were scanned for potential effector binding elements (EBEs) for TAL2. The results revealed that indeed the EBEs were present in respective S gene promoters (Figure 4-4). For Glyma20g04880, the predicted EBE is 294bp upstream from the ATG, while for Glyma05g35190, the EBE is 70bp upstream from the ATG.
Summary and discussion

Six TAL effector genes were identified in Xag12-2. The genes range in repeat number of 15 to 23. Each gene has a unique arrangement of RVDs. TAL effector gene mutagenesis assay indicated that TAL2 seems to be the major virulent one, since only Tal2 mutant strain rendered reduced pustule formation and less population size compared to the wild type strain. However, the Tal2 mutant strains must be complemented with the gene and tested for virulence before any conclusions are made.

A gene (Glyma20g04880) encoding a member of zinc-finger-homeodomain (ZF-HD) transcription factors was highly induced and showed CHX independent expression. A candidate EBE for TAL2 was identified in the promoter region of Glyma20g04880. A relationship between members of LBD and bHLH has been illuminated that AtLOB was capable of interacting with members of the bHLH family, and the binding of bHLH048 to LOB led to a reduction of LOB affinity to DNA (Husbands et al., 2007). Moreover, a connection between ZF-HD and bHLH genes was illustrated in Arabidopsis as well. Three bHLH genes were induced by ectopic expression of the ZF-HD gene ZFHD1, and also were shown to contain the consensus binding sequences for ZFHD1 (Tran et al., 2007). Therefore, further analysis of TAL2 and the candidate target genes are warranted.

Another candidate S gene Glyma05g35190 was annotated as an aluminum activated malate transporter (ALMT) gene. Proteins of this transporter family are mainly involved in aluminum resistance and regulating stomatal movement (Collins et al., 2008; De Angeli et al., 2013a; De Angeli et al., 2013b; Gruber et al., 2010; Hoekenga et al., 2006; Ligaba et al., 2006; Ligaba et al., 2012; Meyer et al., 2010; Meyer et al., 2011; Sasaki et al., 2004). The contribution of ALMT to SBP remains to be clarified. However, if it is identified as the S gene of TAL2, a brand new type will be added into the established S gene pool of transporters, which currently only includes sugar transporter genes (SWEET genes) and a
sulphate transporter gene (Antony et al., 2010a; Cernadas et al., 2014; Yang et al., 2006; Zhou et al., 2015).

Further work is still needed to confirm the two candidate S genes as targets of TAL2. In pepper bacterial leaf spot, upa20 encoding a member of bHLH transcription factor family was identified as the S gene targeted by TAL effector AvrBs3 from the allied species X. campestris pv. vesicatoria (Synonym X. axonopodis pv. vesicatoria). UPA20 was revealed to play a role in cell enlargement, which was proposed to be critical for the induction of hypotrophy during the pathogenesis (Kay et al., 2007). CsLOB1, a member of LBD transcription factor genes, was determined to be the targeted S gene of TAL effector PthA4, PthAw and PthA* from the related species of X. axonopodis pv. citri (Hu et al., 2014; Pereira et al., 2014). Induction of CsLOB1 was critical for the pustule formation (Hu et al., 2014). In SBP pathosystem, both cell enlargement (hypertrophy) and cell multiplication (hyperplasia) were shown to be involved in pustule formation in the infected plant tissues by X. axonopodis pv. glycines strains (Jones et al., 1985; Jones et al., 1987). However, from the RNA-Seq assay, candidates from families of LBD or bHLH transcription factors were not detected.

TAL effectors are commonly present in various Xanthomonas strains and contribute to virulence. AvrBs3, one of the earliest characterized TAL effector members from X. campestris pv. vesicatoria was shown to serve as an apparent virulence factor when S gene upa20 is induced in the host (Bonas et al., 1989; Kay et al., 2007). For the citrus bacterial canker causal strains, many of them carry more than one TAL effector gene. However, only one gene in each strain is known to be the major virulence effector (Al-Saadi et al., 2007; Jalan et al., 2013; Sun et al., 2004; Verniere et al., 1998). Whether TAL effectors play roles as virulence factors in other Xanthomonas-associated diseases is unknown. An understanding of the mechanisms of TAL effector mediated pathogenesis on hosts will be gained by exploring more pathosystems.
Figure 4-1 TAL effector profile of strain Xag12-2.

A. Southern blot results of TAL effector genes of Xag12-2. Hybridization probe is the SphI segment of TAL effector gene *avrXa7*. B. The repeats of the six TAL effectors in Xag12-2. C. The repeat alignment of sequenced TAL effectors of AvrXg1 and TAL2. The differences in the repeats are shown in red highlight.
A. Gel electrophoresis with bands labeled S12 and WT, showing fragment sizes 2799bp and 1953bp.

B. Images of leaf surfaces, labeled 1 to 4, with scale bars indicating 0.2 cm and 0.1 cm.

C. Bar chart showing average number of pustules with WT and S12 treatments, with error bars and a significance marker (*) indicating a difference between groups.

D. Graph plotting log10 colony-forming units (cfu) per cm² over days post-infection (dpi), with markers (*), blue line for WT and orange line for S12.
Figure 4-2 Strain virulence is reduced in a Tal2 mutant.

A. Southern hybridization analysis of the Tal2 mutant strain. Samples in each lane are shown above the blot picture. The right lane is the marker with the sized labeled on the right. B. The effect of TAL2 on the disease symptoms. Soybean leaves were inoculated with S12 (1 and 3) or WT (2 and 4). Pictures were taken for the inoculated sites at 5 dpi by dissecting microscope at 2X magnification (1 and 2) and 6.4X magnification (3 and 4). C. Number of pustules counted for each inoculation sites (0.20cm²) at 5dpi. D. Bacterial population assays of two strains WT and S12 on Spencer cultivar. X-axis shows the days post inoculation. Asterisks (*) indicate significant difference between the two treatments according to t-test ($P < 0.05$). Inocula consisted of bacterial suspensions of a $10^3$ dilution of $OD_{600}$=0.2. WT: Xag12-2 wild type; S12: Xag12-2 Tal2 mutant.
Table 4-1 Top ten up-regulated genes by TAL2 from RNA-Seq data analysis.

Samples are RNA from soybean leaves treated with two strains with and without CHX. Samples were taken at 24 hpi. Inocula were bacterial suspension of OD$_{600}$=0.2. WT: Xag12-2 wild type; S12: Xag12-2 Tal2 mutant.

<table>
<thead>
<tr>
<th>Gene ID (Phytozome)</th>
<th>Treatment</th>
<th>log2 (fold change WT/S12)</th>
<th>q value</th>
<th>Annotation</th>
<th>Treatment with Cyclohexmide (100mg/ml)</th>
<th>log2(fold change WTC/S12C)</th>
<th>q value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyma20g04880</td>
<td>WT 28.27</td>
<td>0.73</td>
<td>5.27</td>
<td>0.00</td>
<td>ZF-HD transcription factor</td>
<td>22.33</td>
<td>0.07</td>
</tr>
<tr>
<td>Glyma19g43940</td>
<td>WT 17.24</td>
<td>0.46</td>
<td>5.24</td>
<td>0.00</td>
<td>GDSL esterase/lipase</td>
<td>0.88</td>
<td>0.07</td>
</tr>
<tr>
<td>Glyma09g25330</td>
<td>WT 146.16</td>
<td>4.39</td>
<td>5.06</td>
<td>0.00</td>
<td>cytokinin hydroxylase-like</td>
<td>7.73</td>
<td>4.46</td>
</tr>
<tr>
<td>Glyma02g42640</td>
<td>WT 73.89</td>
<td>2.29</td>
<td>5.01</td>
<td>0.00</td>
<td></td>
<td>9.39</td>
<td>0.45</td>
</tr>
<tr>
<td>Glyma14g39670</td>
<td>WT 36.30</td>
<td>1.15</td>
<td>4.99</td>
<td>0.00</td>
<td>FRUCTOKINASE</td>
<td>1.31</td>
<td>0.01</td>
</tr>
<tr>
<td>Glyma20g32080</td>
<td>WT 10.85</td>
<td>0.35</td>
<td>4.94</td>
<td>0.00</td>
<td>NPH3 family</td>
<td>0.36</td>
<td>0</td>
</tr>
<tr>
<td>Glyma03g41100</td>
<td>WT 70.76</td>
<td>2.31</td>
<td>4.94</td>
<td>0.00</td>
<td>Myb-like DNA-binding domain</td>
<td>5.57</td>
<td>0.84</td>
</tr>
<tr>
<td>Glyma20g29011</td>
<td>WT 27.40</td>
<td>0.91</td>
<td>4.91</td>
<td>0.00</td>
<td>LEUCINE-RICH REPEAT RECEPTOR-LIKE PROTEIN KINASE</td>
<td>15.22</td>
<td>0.27</td>
</tr>
<tr>
<td>Glyma13g40670</td>
<td>WT 6.02</td>
<td>0.21</td>
<td>4.87</td>
<td>0.00</td>
<td>3-Oxoacyl-[acyl-carrier-protein (ACP)] synthase III C terminal</td>
<td>0.37</td>
<td>0</td>
</tr>
<tr>
<td>Glyma05g35190</td>
<td>WT 60.39</td>
<td>4.77</td>
<td>3.66</td>
<td>0.00</td>
<td>Aluminium activated malate transporter</td>
<td>64.85</td>
<td>4.82</td>
</tr>
</tbody>
</table>
Figure 4-3 RNA expression assay of soybean leaves treated with WT or S12 with or without CHX. Samples were taken at 24 hpi. Inocula were bacterial suspension of OD$_{600}$=0.2. WT: Xag12-2 wild type; S12: Xag12-2 Tal2 mutant. WTC and S12C are WT and S12 with CHX. Error bars indicate standard deviations for three independent experimental replicates. Asterisks (*) - indicate significant difference between two samples in the same bracket according to t-test ($P < 0.05$).
Figure 4-4 Potential EBEs in promoters of Glyma20g04880 and Glyma05g35190.

The sequences represents the respective promoter regions for Glyma20g04880 and Glyma05g35190. The TAL effectors are represented by the repeat regions in which a single letter indicates each RVD. (I-NI; G-NG; S-NS; D-HD, *-N*). The repeat regions align with the putative EBEs. The red letters indicate the optimized match between the RVDs and the corresponding nucleotides. The red ATG indicates the initiation codon, yellow highlights the predicated TATA boxes.
References


bacterial blight and a novel susceptibility gene. *Plos Pathogens, 10*(2), e1003972. doi:10.1371/journal.ppat.1003972


Hentrich, M., Sanchez-Parra, B., Perez Alonso, M., Carrasco Loba, V., Carrillo, L., Vicente-Carbajosa, J., et.al. (2013). YUCCA8 and YUCCA9 overexpression reveals a link between auxin signaling and lignification through the induction of ethylene biosynthesis. Plant Signaling & Behavior, 8(11), e26363-e26363. doi:10.4161/psb.26363


Hoekenga, O. A., Maron, L. G., Piñeros, M., Cançado, G., Shaff, J., Kobayashi, Y., et al. (2006). AtALMT1, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America, 103(25), 9738-9743. doi: 10.1073/pnas.0602868103


Jiang, G. H., Xia, Z. H., Zhou, Y. L., Wan, J., Li, D. Y., Chen, R. S., et al. (2006). Testifying the rice bacterial blight resistance gene *xa5* by genetic complementation and further analyzing *xa5 (Xa5)* in...


Proceedings of the National Academy of Sciences of the United States of America, 109(47), 19480-19485. doi:10.1073/pnas.1212415109


Yang, Y. N., Defeyter, R., & Gabriel, D. W. (1994). Host-specific symptoms and increased release of *Xanthomonas citri* and *Xanthomonas campestris* pv. *malvaeearum* from leaves are determined by the 102-bp tandem repeats of *pthA* and *avrb6*, respectively. *Molecular Plant-Microbe Interactions, 7*(3), 345-355. doi:10.1094/MPMI-7-0345


Appendix A - Strain, plasmids and primers used in the projects

Table A-1 Strains and plasmids used in the study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Feature</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xanthomonas citri</em> subsp. <em>citri</em></td>
<td>Xcc306 Group A, wild-type, Rifr</td>
<td>DPI*</td>
</tr>
<tr>
<td>Xcc306WT</td>
<td>Xcc306 Group A, wild-type, Rifr</td>
<td></td>
</tr>
<tr>
<td>Xcc306ΔpthA4</td>
<td>pthA4 deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>Xcc306ΔpthA4::pthAw</td>
<td>pthAw complement Xcc306 ΔpthA4, Gm</td>
<td>This study</td>
</tr>
<tr>
<td>Xcc306ΔpthA4::dCsSWEET1</td>
<td>Artificial TAL effector targeting CsSWEET1 complement Xcc306 ΔpthA4, Gm</td>
<td>This study</td>
</tr>
<tr>
<td>Xcc306ΔpthA4::dCsLOB1</td>
<td>Artificial TAL effector targeting CsLOB1 complement Xcc306 ΔpthA4, Gm</td>
<td>This study</td>
</tr>
<tr>
<td>Xcc306ΔpthA4::dCsLOB2</td>
<td>Artificial TAL effector targeting CsLOB2 complement Xcc306 ΔpthA4, Gm</td>
<td>This study</td>
</tr>
<tr>
<td>Xcc306ΔpthA4::dCsLOB3</td>
<td>Artificial TAL effector targeting CsLOB3 complement Xcc306 ΔpthA4, Gm</td>
<td>This study</td>
</tr>
<tr>
<td>Xcc306ΔpthA4::dCsLOB4</td>
<td>Artificial TAL effector targeting CsLOB4 complement Xcc306 ΔpthA4, Gm</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Xanthomonas campestris pv. Vesicatora**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xcv8510</td>
<td>Wild-type</td>
<td>Jeffery Johne’s lab (Gainesville, FL)</td>
</tr>
<tr>
<td>Xcv8510::PHM</td>
<td>Empty vector PHMI complement</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xcv8510</td>
<td></td>
</tr>
<tr>
<td>Xcv8510::dCsLOB2</td>
<td>Artificial TALE targeting</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CsLOB2 complement</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Xcv8510, Gm</td>
<td></td>
</tr>
</tbody>
</table>

**Xanthomonas axonopodis pv. glycines**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xag12-2</td>
<td>A wild type strain</td>
<td>This study</td>
</tr>
<tr>
<td>S12</td>
<td>A Xag12-2 Tal2 mutant strain</td>
<td>This study</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F−recAφ80dlacZΔM15</td>
<td>NEB</td>
</tr>
</tbody>
</table>

**Escherichia coli**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript KS(+)</td>
<td>Phagemid, pUC derivative,</td>
<td>Stratagene</td>
</tr>
<tr>
<td></td>
<td>Amp, PthAw</td>
<td></td>
</tr>
<tr>
<td>PHMI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUFR053</td>
<td>repW, Mob+, LacZα+, Par+, Gmr</td>
<td>Clontech</td>
</tr>
<tr>
<td>PZW-pthAw</td>
<td>pBluescript KS(+) with pthAw gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

Amp, ampicillin; Gm, gentamicin; Sp, spectinomycin; Rif, rifamycin;

*DPI, Division of Plant Industry of the Florida Department of Agriculture and Consumer Services (Gainesville, FL).
†BRL, Bethesda Research Laboratories (Gaithersburg, MD).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOB-RT-F</td>
<td>CTCACTAAACTACTACAACCCAACAGTG</td>
<td>qRT-PCR for <em>CsLOB1</em> gene</td>
</tr>
<tr>
<td>LOB-RT-R</td>
<td>GCATGGAGAAGATTGAGAAGGAGG</td>
<td></td>
</tr>
<tr>
<td>CORT-F</td>
<td>AAGATTCTCAAGGGCAGCCTGTCTCTGGAGAGAT</td>
<td>qRT-PCR for transcript</td>
</tr>
<tr>
<td>CORT-R</td>
<td>TCTGGTGGCTGTGGGATGCTGAGAT</td>
<td>orange1.1g001725m</td>
</tr>
<tr>
<td>45080-RP-F</td>
<td>CCCTCTTTTCTTCTCCTCTTCTCTCT</td>
<td>qRT-PCR for <em>CsLOB2</em> gene</td>
</tr>
<tr>
<td>45080-RP-R</td>
<td>AAGCTGCGCATGGACTAA</td>
<td></td>
</tr>
<tr>
<td>47491-RP-F</td>
<td>GCCACAAGGAATCCTAGCAAAAG</td>
<td>qRT-PCR for <em>CsLOB3</em> gene</td>
</tr>
<tr>
<td>47491-RP-R</td>
<td>CAGCACAAGGGCTCATGATA</td>
<td></td>
</tr>
<tr>
<td>36782-RT-F1</td>
<td>CCCAGATGACAAAGTCGTCTCTCTCTCTCCTCTCT</td>
<td>qRT-PCR for <em>CsLOB4</em> gene</td>
</tr>
<tr>
<td>36782-RT-R1</td>
<td>CTCTCTCTCTTGTGGAGGGAGTCAT</td>
<td></td>
</tr>
<tr>
<td>Cit.2392-RP-F</td>
<td>GTTGGTGCTGTCTGTTTCTCTTC</td>
<td>qRT-PCR for transcript</td>
</tr>
<tr>
<td>Cit.2392-RP-R</td>
<td>AGACAGATTCATGCCAATCCTCTCTCTCTCTCTCT</td>
<td>orange1.1g010632m</td>
</tr>
<tr>
<td>Cit.7877-RP-F</td>
<td>GAGGCAACAGTTCATTGTGTGTA</td>
<td>qRT-PCR for transcript</td>
</tr>
<tr>
<td>Cit.7877-RP-R</td>
<td>GGATTGAGACCAACACATGAG</td>
<td>orange1.1g023962m</td>
</tr>
<tr>
<td>Cit.20509-RP-F</td>
<td>GGCTCTCTGGTGCTGACATCTA</td>
<td>qRT-PCR for transcript</td>
</tr>
<tr>
<td>Cit.20509-RP-R</td>
<td>GACTGGAAGCAGAAAAGGTCTAA</td>
<td>orange1.1g015623m</td>
</tr>
<tr>
<td>Cit.8700-RP-F</td>
<td>CACCCTACGTCTATGCATCTCTCTCTCTCTCTCTCT</td>
<td>qRT-PCR for transcript</td>
</tr>
<tr>
<td>Cit.8700-RP-R</td>
<td>GGATACCATTAGCCTCTCCATAAA</td>
<td>orange1.1g032298m</td>
</tr>
<tr>
<td>Cit.30858-RP-F</td>
<td>CAAAAGCGCTCATGCTACATTTC</td>
<td>qRT-PCR for transcript</td>
</tr>
<tr>
<td>Cit.30858-RP-R</td>
<td>GGTGTTAACCCGCTATCCTTT</td>
<td>orange1.1g024916m</td>
</tr>
<tr>
<td>Primers</td>
<td>Forward Sequence</td>
<td>Reverse Sequence</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Cs4g07290-RT-F</td>
<td>GTAGTGAAGGTTTCGCAGAATG</td>
<td>TTGATGCTGGCCAAATGAG</td>
</tr>
<tr>
<td>Cs4g07290-RT-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cs8g16780-RT-F</td>
<td>TTGGTGAGATGGTGTGAG</td>
<td>CTTCAGGTCTGGTACCAATGAA</td>
</tr>
<tr>
<td>Cs8g16780-RT-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZF-HD-RT-F</td>
<td>CCCGATATCAAGCCTCAGAATG</td>
<td>TCAGAGGGTGAGGAGAATA</td>
</tr>
<tr>
<td>ZF-HD-RT-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALMT-RT-F</td>
<td>CCT ATG CAA AGC TCT ACC CTA C</td>
<td>CCT GTG CTA TAC CCA GAT ACA G</td>
</tr>
<tr>
<td>ALMT-RT-R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>