To my parents who always understand and support me in chasing my dream
ACKNOWLEDGMENTS

I would like to express my heartfelt thanks to all the people who helped and inspired me during my master’s study. I want to thank the chair of my academic committee and advisor Dr. Gloria Moore for her financial support and dedicated advisory during my entire study. Specially thank her for always being supportive even at the most difficult time studying in this foreign country, without her trust I would not had been able to achieve this milestone. Dr. Moore’s kindness and easygoing personality will remain in me and guide me to treat other people like she does.

My gratitude also goes to my committee members Dr. Vicente Febres and Dr. Jeffrey Jones. I am very delighted to interact with them and have learned tremendous knowledge from them. Their insights to plant pathology explain to me what world-class scientists would be like. Repetitive thanks given to Dr. Febres not only because his tireless scientific guidance to me, working with him on a daily basis, he spent large amount of precious time teaching me laboratory skills and taking care of me in my everyday life. I feel happy and proud to have such a friend and spiritual father.

I would also like to thank Dr. Abeer Khalaf for her patient explanations and encouragement, and thanks to Kimberly, Latanya, Terry and Fabiana for their help and working with them was really a pleasure. Last but not least, deep thanks go to my parents far back in China for their understanding and support, and thanks to my wife for being with me and taking care of my life. I love you all.
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<td>AzA</td>
<td>Azelaic Acid</td>
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<tr>
<td>ETI</td>
<td>Effector-Triggered Immunity</td>
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<td>HLB</td>
<td>Huanglongbing</td>
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<td>MES</td>
<td>2-(N-morpholino) ethanesulfonic acid</td>
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<td>PAMP</td>
<td>Pathogen-Associated Molecular Pattern</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PTI</td>
<td>PAMP-Triggered Immunity</td>
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<td>SA</td>
<td>Salicylic Acid</td>
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<td>SAR</td>
<td>Systemic Acquired Resistance</td>
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<td>Xcc</td>
<td><em>Xanthomonas citri</em> subsp. <em>citri</em></td>
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The citrus industry plays an important role in the economy of Florida. However, bacterial diseases such as citrus canker (caused by *Xanthomonas citri* subsp. *citri*, *Xcc*) and huanglongbing (caused by *Candidatus Liberibacter asiaticus*, *Las*) have caused considerable losses to citrus production. To have a better understanding of the defense response of citrus against *Xcc* and *Las* and to seek alternative ways to control canker and huanglongbing, we studied: 1) The role of Pathogen-Associated Molecular Pattern (PAMP)-triggered immunity (PTI) in the response of resistant kumquat and susceptible grapefruit, to *Xcc*; 2) The effect of PAMPs from *Xcc* or *Las* on PTI; and 3) Evaluated the effect of azelaic acid, a molecule recently found to be important in the priming and signaling of pathogen defense, on the PTI response in grapefruit.

The first experiment was performed by treating kumquat and grapefruit with a 22 amino acid peptide from the *Xcc* bacterial flagellin conserved domain (Xflg22), a PAMP. The results showed that, compared to the mock inoculation, Xflg22 (10 µM) triggered the expression of a set of defense-associated genes including EDS1, RAR1, SGT1, EDR1, PBS1, NDR1, EDS5, PAL, NPR2, NPR3, RdRP and AZI1 in kumquat, but the corresponding genes in grapefruit were either not induced or showed slight
downregulation. In kumquat, the significantly Xflg22 induced genes EDS1, RAR1, SGT1 and NDR1 are thought to be PTI-associated genes, suggesting that Xflg22 initiated PTI in this resistant species. However, no obvious induction of those genes was observed in grapefruit suggesting a weaker PTI and perhaps part of the reason this species is susceptible to canker.

In the second experiment we compared the effect of Xflg22 with the flg22 derived from Las (Lflg22) as well as the archetype flg22 on grapefruit. Additionally, azelaic acid was used as a pretreatment before any flg22 infiltration in order to evaluate its defense-eliciting capability in citrus. Comparison between the effects of the three different flg22s showed that most of the defense-associated genes tested were not differentially expressed between the treatments compared with the mock inoculation, except for salicylic acid signaling gene AZI1 and salicylic acid biosynthesis gene PAL. However, when plants were pretreated with azelaic acid (1 mM) two days prior to the PAMP treatments, mock inoculation, flg22 and Xflg22 treatments all showed higher expression levels of EDS1, RAR1, EDR1, EDS5, NPR1, NPR2, NPR3, RdRP and AZI1 than that of in control buffer pretreated plants, except the Lflg22 treatment which significantly reduced the expression of EDS1, RAR1, SGT1, EDR1, NPR1, NPR2 and NPR3 compared with the mock inoculation. Azelaic acid alone induced a set of defense-associated genes including EDS1, RAR1, EDR1, EDS5, NPR1, NPR2, NPR3, RdRP and AZI1, which was comparable with the gene inductions by Xflg22 in kumquat, suggesting that azelaic acid could be used as a potential chemical to control bacterial disease in citrus.
CHAPTER 1
INTRODUCTION

With hundreds of years of growing history, citrus production in Florida has become a multibillion dollar industry contributing most of the value to Florida’s agriculture (http://www.florida-agriculture.com/agfacts.htm). Citrus production in Florida accounted for 65 percent of the total citrus market in the United States during the 2009-2010 season, far more than that of California (31%), Texas and Arizona (4% combined) (http://www.nass.usda.gov/Statistics_by_State/Florida/Publications/Citrus/fcs/2009-10/fcs0910.pdf). Multiple citrus types are grown in Florida, among which grapefruit (Citrus paradise Macf.) is one of the most popular. It is consumed as fresh fruit and juice not only because it is flavorful but it is also a good sources of health promoting nutrients and phytochemicals such as vitamin C, antioxidants and fiber pectin [1,2].

As the largest grapefruit producing state in the United States, Florida is suffering from a serious disease, citrus canker, caused by the bacterium Xanthomonas citri subsp. citri (Xcc). Grapefruit is highly susceptible to citrus canker and the disease causes lesions on leaves, stems and fruits. Fruit lesions make them cosmetically deficient and devalue the commodity. When favorable conditions such as high winds and rainfall are present, severe infection can lead to attenuated tree vigor characterized by defoliation, death of young shoots, and fruit drop [3]. In an effort to eliminate canker in Florida, the U.S. Department of Agriculture (USDA) in cooperation with Florida Department of Agriculture and Consumer Service (FDACS) declared an eradication program in 1994, mandating the removal of diseased and surrounding trees, including in backyards. However, the hurricanes in 2004 exacerbated the spread of canker making the eradication unrealistic. As a result, the USDA withdrew the eradication program and

Several management methods can be used to control canker with various degrees of effectiveness. Since wind-blown rain facilitates canker bacteria dissemination, windbreaks in the infected orchard can restrict disease progress by reducing wind speed [4]. The Asian leafminer (*Phyllocnistis citrella*) larvae produce wound galleries in the citrus leaves and make them more susceptible to canker, so either chemical or biological control of the leafminer is an indirect way to manage citrus canker spreading [3]. In terms of bactericide use, copper-based sprays so far are more effective than any other antibacterial chemicals tested [5,6]. However, the antibacterial activity is limited to the surfaces of leaves or fruit and multiple spray times are needed to reach the optimal effect [3,6].

In Florida, huanglongbing (or citrus greening), caused by *Candidatus Liberibacter asiaticus*, is another bacterial disease affecting the citrus industry. Transmitted by the vector psyllid (*Diaphorina citri* Kuwayama), bacterial infection can result in small and misshapen fruits and largely reduced yield and lifespan of citrus trees (http://www.aphis.usda.gov/plant_health/plant_pest_info/citrus_greening/background.shtml). Because the disease has long latency, nonspecific and environment dependant symptoms, the efficient detection and the management of huanglongbing is difficult [7]. Almost all the cultivated citrus species and citrus relatives can be infected by huanglongbing. The current methods for controlling huanglongbing are through chemical control of psyllid vector, removal of infected trees and using disease-free nursery materials [8,9].
Facing the threat of new diseases in Florida, it is necessary to study these diseases. Studying the citrus defense response at the molecular level can be helpful to understand the reasons for the vulnerability to their bacterial pathogens. One of the supportive evidences for that is the genomic sequencing of model plants and pathogenic bacteria has provided a better understanding of the plant immune system and plant-microbe interactions at the molecular, genetic and evolutionary levels [10,11]. With little understanding of the citrus-pathogen system, taking advantage of the latest information on plant immunity and plant-pathogen interactions and combining with the recent availability of a citrus genome database, we intend to explore mechanisms of citrus susceptibility and resistance to its bacterial diseases and seek alternative effective management methods.
CHAPTER 2
LITERATURE REVIEW

The Plant Immune System

Plants use two different pathways of active defense or immunity against potential pathogens to prevent the establishment of disease [12]. In one pathway plants respond to certain molecules that originate from phytopathogenic microorganisms. These are named Pathogen-Associated Molecular Patterns (PAMPs). Recognition of PAMPs in the plant is mediated by pattern recognition receptors (PRRs), which triggers signaling cascades that can lead to a defense response [13,14]. The PAMP triggered immunity (PTI) in plants is also termed the basal defense and it deters colonization by a broad range of microbes [15]. Pathogens that successfully overcome the plant PTI may further induce a stronger defense response, effector-triggered immunity (ETI), which brings about disease resistance in the plant. To be specific, plant resistance (R) genes encode proteins mostly with nucleotide binding (NB) and leucine rich repeat (LRR) domains that recognize pathogen virulence factors (effectors), resulting in the initiation of R-mediated resistance. ETI is typically accompanied by the hypersensitive response (HR) [12,16], a programmed cell death process effectively restraining pathogen growth at the infection site where lesions serve as visual markers.

Additionally, when infected by pathogens, plants are not only able to generate defense mechanisms locally but also to transduce signals to distal plant tissues triggering systemic resistance to protect against subsequent pathogen attacks. This process is termed Systemic Acquired Resistance (SAR) [17,18]. One of the examples is that Arabidopsis thaliana R-mediated resistance at the infection site leads to elevated systemic protection against virulent bacteria [19].
PAMPs and PAMPs-Triggered Immunity (PTI)

Plant basal defense triggered by PAMPs is essential, without it plants would be vulnerable to even minor microbial challenges. As conserved molecules among microbial species, PAMPs such as flagellin and the amino-terminus of the elongation factor Tu (EF-Tu) are required for the perception of bacteria in plants [20,21]. For plant perception of fungi and oomycetes, two known PAMPs are chitin and ergosterol [14,22,23]. Flagellin is a component of the bacterial flagellum filaments and it contains a conserved 22 amino acids (flg22) domain that is sufficient to be recognized by a LRR receptor-like kinase (FLAGELLIN SENSING 2, FLS2) in plants, which activates a mitogen-activated protein kinase (MAPK) cascade that results in the regulation of defense-related genes [24,25,26] (Figure 2-1). In *Arabidopsis*, flagellin treatment can result in cell death and plant developmental impediment, and this effect depends on the amino acid residue at position 43 (aspartic acid) on the flagellin protein [27]. *Arabidopsis* plants treated with flg22 produce a microRNA that degrades mRNAs of several auxin signaling components, halting the growth of phytopathogenic bacteria [28]. Antagonistic to auxin signaling, SA accumulates during flg22 infection and mutation of certain SA signaling genes leads to dampened PTI, indicating that an SA-mediated defense response is one consequence of flg22 perception [29,30]. Furthermore, ion fluxes change, such as calcium ions increase [31], synthesis of callose [32] and stomatal closure [33] also contribute to PTI.
Figure 2-1. Simplified model of bacteria flagellin perception in *Arabidopsis*. A conserved 22 amino acid sequence in the bacterial flagellin is recognized and bound by a LRR containing receptor (FLS2), which also has kinase activity that triggers a MAPK signaling cascade [26,34]. Multiple MAP kinases, MAP kinase kinases and MAP kinase kinase kinases are involved in the regulation of WRKY transcription factors, resulting in the modulation of the transcription of defense genes and PTI [24].

**Defense Signals**

The correlation between induction of SAR and accumulation of SA in plants upon pathogen infection [35], along with the evidence that exogenous treatment with SA appears to initiate expression of SAR-related genes implies that SA may be the chemical involved in SAR signal transduction [36,37]. However, pathogen infected tobacco rootstocks engineered with a bacterial SA-degrading enzyme NahG are still capable of transmitting SAR signals to the wild type scions and only accumulation of SA in distant tissues is necessary for SAR induction [38].
A subsequent study in the tobacco-Tobacco Mosaic Virus (TMV) system indicates that methyl salicylate (MeSA) was actually a key SAR signal component since MeSA could move from local infection sites to distal healthy leaves and was then hydrolyzed into SA by salicylic acid-binding protein 2 (SABP2), which triggered downstream defense signaling that established resistance systemically [39,40]. Nevertheless, it is still too early to conclude that MeSA is the universal SAR inducing signal because similar research in Arabidopsis demonstrates that MeSA is not as effective in triggering SAR as was shown in the tobacco-TMV system [41].

A study of vascular sap extracted from Arabidopsis infected with a systemic resistance inducible pathogen revealed that azelaic acid, a nine-carbon dicarboxylic acid, had higher accumulation in SAR-induced exudates than in mock-induced exudates [42]. The increase of azelaic acid was found to be involved in the onset of SA accumulation and exogenous application of this chemical induced an earlier and stronger defense response both locally and systemically. Meanwhile, the AZI1 (AZELAIC ACID INDUCED 1) gene, encoding a putative secreted protease inhibitor/seed storage/lipid transfer protein family protein, was induced by azelaic acid and mutation of this gene resulted in attenuated SAR. Taken together, mediated through AZI1, azelaic acid was an important signal involved in priming the plant defense [42].

The elevation of jasmonic acid (JA) content and heightened expression levels of JA biosynthetic genes observed when Arabidopsis leaves were inoculated by SAR inducing avirulent Pseudomonas syringae, plus the evidence that JA treated plants are more competent in systemic resistance, gave the cue that JA was also a good
candidate signal for initiating SAR [43]. However, instead of using low inoculum concentration of pathogen, a parallel experiment challenged plants with an HR-eliciting concentration of bacteria suggested that JA signaling components were not always required, and JA accumulation did not positively correlated with SAR activity [41,44], implying that the involvement of JA and its signaling in SAR depends on pathogen pressure [45].

**Genes Involved in Plant Immunity**

**Enhanced Disease Susceptibility 1 (EDS1) and Nonrace-Specific Disease Resistance 1 (NDR1)**

Mutant *eds1* *Arabidopsis* plants were found to have both attenuated basal resistance against a non-host pathogen and R proteins mediated resistance [46,47]. Biochemical analysis demonstrated that the R proteins involved were characterized by their Toll-Interleukin-1 receptor (TIR) and nucleotide binding-leucine rich repeat (NB-LRR) domains [48]. EDS1 functions mainly by association with proteins encoded by phytoalexin deficient4 (PAD4) or senescence-associated gene101 (SAG101), forming a protein complex coordinating signaling events delivered by ETI and PTI [49,50,51]. It is believed that regulation by the EDS1 complex occurs immediately downstream of R-protein activation but upstream of both SA accumulation and the oxidative burst that leads to HR [51,52]. In parallel with EDS1, NONRACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) was found to be a regulator gene whose protein is associated with a different class of R proteins (coiled-coil motif containing) responding to specific avirulent proteins. Like EDS1, NDR1 is proposed to be involved in basal resistance to both compatible and non-host pathogens [53]. The *Arabidopsis ndr1* mutant exhibits decreased SA production and SAR and NDR1 regulation was shown to be upstream of
SA synthesis but downstream of reactive oxygen species (ROS) production, which potentially leads to programmed cell death [48,54,55].

**Requires for Mla12 Resistance (RAR1) and Suppressor of G-TwoAlleleofSkp1 (SGT1)**

With the concept that R protein-effector interaction is mediated by third party protein complexes [56], RAR1 and SGT1 were characterized as important components coordinating R-mediated resistance [57,58]. RAR1 mutated barley and *Arabidopsis* both showed compromised resistance specifically triggered by R genes [59,60], and similar weakened R gene-mediated resistance was also observed in SGT1 silenced barley, tobacco and *Arabidopsis* [61,62,63]. The binding between RAR1 and SGT1 proteins was confirmed *in vitro* [61] and another molecular chaperone Heat Shock Protein 90 (HSP90) was also found to be part of the RAR1-SGT1 complex [64]. However, R-mediated resistance does not always require the physical interaction between these three proteins [57]. Noticeably, SGT1 silenced *Nicotiana benthamiana* plants inoculated with a non-pathogen microbe accumulated a higher bacterial population than that of wild type inoculated plants and an *Arabidopsis* RAR1 mutation lead to subdued basal defense against both compatible and non-host pathogens [62]. Similarly, in soybean RAR1 and SGT1 were found to be converging signaling components for basal defense [65]. These findings indicate that RAR1 and SGT1 are not only essential regulators of R gene-mediated defense but are necessary for plant basal defense against a wide range of microbes.

**Enhanced Disease Resistance (EDR1)**

EDR1 is a negative regulator of the plant defense response and it has been shown that *Arabidopsis edr1* mutants have enhanced resistance to the powdery mildew fungus
Erysiphe cichoracearum [66]. EDR1 mediated defense is dependent on SA signaling pathways and do not require JA or ethylene signaling directly [67,68,69]. However, exogenous application of ethylene to edr1 mutants can cause faster senescence followed by increased expression of PR genes, the marker of SAR, implying that EDR1 may regulate defense response through the senescence process [70,71]. Biochemical analysis revealed that EDR1 encodes a putative Raf family MAP kinase kinase kinase (MAPKKK) having an autophosphorylation activity, which negative regulates the defense response [70,72].

Enhanced Disease Susceptibility 5 (EDS5)

When inoculated with pathogenic bacteria, susceptible Arabidopsis mutant eds5 shows failure to accumulate SA and a reduced transcriptional level of the Pathogenesis Related 1 gene (PR1), which is one of the markers of SAR. However, the susceptibility observed in the eds5 mutant was not as strong as in plants expressing SA degrading enzymes (NahG), which had attenuated transcription of multiple PR genes (PR-1, PR-2, PR-5) [73,74]. When a cloned EDS5 was used to complement the eds5 mutant the resulting plant showed accumulation of SA and expression of PR-1, implying that EDS5 regulated the defense response upstream of SA synthesis and SAR onset [75]. What is more, eds1, pad4 and ndr1 mutants exhibit significantly decreased EDS5 transcription levels upon pathogen inoculation, indicating that EDS5 functions downstream of these signaling components.

Pathogenesis-Related Gene 1 (PR1) and Nonexpressor of PR Genes (NPR1)

PR1 gene expression is considered a marker of SAR given the evidence that its expression correlates with the initiation of SAR and that PR1 is the most highly induced
PR gene after a plant has been inoculated with SAR-inducing pathogens or treated with SA. However, the function of the PR1 protein is still unclear [76,77].

NPR1 plays a key role as a transcriptional activator for the PR1 gene [78]. The Arabidopsis npr1 mutant is not able to respond to the SAR eliciting chemical SA and fails to induce multiple PR genes including PR1 [79,80,81]. Cytoplasmic NPR1 exists in anoligomeric form and, upon infection, SA-mediated cellular redox changes cause the NPR1 oligomers to break into monomers, which are then translocated into the nucleus [82]. Nuclear localization of NPR1 is necessary to trigger PR1 expression [83] and a bipartite nuclear localization signal (NLS) in the NPR1 protein is necessary for its translocation to the nucleus [84]. Yeast two-hybrid assays suggest that functional NPR1 binds to the TGA family of transcription factors, which are regulators of the transcription of PR1 by binding to its SA-responsive element in the promoter region [78,83,85]. Moreover, NPR1’s transactivation activity is in turn regulated by a proteasome-mediated mechanism: in uninfected plants, nuclear NPR1 is subjected to a cullin3-based ubiquitin ligase-mediated degradation process to avoid constitutive expression of the PR gene; When the plant is challenged with a pathogen, phosphorylation of NPR1 at the Ser11/Ser15 residues also led to proteolysis of NPR1, probably to stimulate an influx of “fresh” NPR1 [86]. In the Arabidopsis genome, there are five NPR1 paralogs that share sequence similarity with NPR1 (NPR2, NPR3, NPR4, BOP1 and BOP2) and the phylogenetic relationships between these paralogs are shown in figure 2-2 [87,88]. Functional analysis indicates that NPR3 and NPR4 also interact with the TGA family of transcription factors and negatively regulate expression of the PR1 gene [87]. The sequences of BOP1 and BOP2 have the least similarity with NPR1 and these proteins
also bind to a TGA of subfamily transcription factor. However, the expression of BOP1 and BOP2 is tissue specific and the gene products regulate plant growth asymmetry in Arabidopsis [88,89].

Azelaic Acid Induced 1 (AZI1)

Arabidopsis AZI1 (At4g12470) is one of the genes induced in cutinase transformed plants that are confirmed to be fully resistant to the fungal pathogen Botrytis cinerea, and overexpression of AZI1 provides disease resistance in Arabidopsis [90]. The protein sequence of AZI1 is homologous to a lipid transfer protein (LTP) family that has the putative functions of fatty acid binding and phospholipid transfer during plant defense against pathogens [91]. The nomenclature of At4g12470 as AZELAIC ACID INDUCED 1 (AZI1) originated because expression of the gene is initiated in azelaic acid triggered resistance and AZI1 can be induced by azelaic acid applications. In addition AZI1 seems to be involved in the regulation or direct translocation of the
SAR signaling to systemic tissues, given the evidence that *azi1* mutants showed normal local resistance but had a compromised systemic resistance [42].

**RNA-Dependent RNA Polymerase (RdRP)**

In the defense against RNA viruses, plants use an RNA silencing mechanism to inhibit viral replication in the tissues, during which a surveillance system of the plant detects exogenous RNA and initiates double-strand RNA (dsRNA) mediated RNA degradation [92]. It has been shown that RNA-dependent RNA polymerases (RdRPs) are a family of proteins necessary for RNA silencing in terms of dsRNA synthesis and silencing signal amplification [93,94,95]. Some RdRPs have been shown to be inducible by either SA or infection with an RNA virus in *Arabidopsis* and *Nicotiana tabacum* [96,97], whereas a mutation in the SA-inducible RdRP gene in *Nicotiana benthamiana* caused the plant susceptible to the viruses [98], which indicates that this particular RdRp is essential for the plant defense against virus and the antivirus response is also associated with the SA-mediated defense response.

**Isochorismate Synthase 1 (ICS1) and Phenylalanine Ammonia Lyase (PAL)**

The synthesis of SA is believed to occur via two parallel pathways [99,100] (Figure 2-3). The enzymatic reactions from chorismate have been shown to be the most important one during pathogen-induced SA biosynthesis in multiple plant-pathogen systems [101,102,103,104]. Silencing the *ics1* gene leads to reduced SA accumulation in tobacco and tomato during pathogen infection and results in a compromised defense response, suggesting ICS1 is a key enzyme for defense-associated SA synthesis [101,102]. On the other hand, a biochemical study using isotope feeding showed that PAL is involved in SA synthesis via cinnamate intermediates and that inhibiting PAL activity decreases SA accumulation induced by a pathogen [105,106]. Moreover, PAL
has been shown to be an important protein catalyzing the first enzymatic reaction in the phenylpropanoid biosynthetic pathway (lignin biosynthesis) [107]. Lignin is considered a necessary physical barrier against non-host bacterial invasion in Arabidopsis [108].

![Figure 2-3. A simplified salicylic acid (SA) synthetic pathway [99]. The key enzymes are indicated above the arrows: PAL is phenylalanine ammonia lyase, ICS1 is isochorismate synthase 1 and IPL is isochorismate pyruvate lyase.](image)

**Avrpphb Susceptible 1 (PBS1)**

PBS1 is an important component for the R-mediated resistance in Arabidopsis against plant pathogenic bacteria (i.e. the resistance induced by the R protein PRS5 recognition of the avrPphB effector secreted from Pseudomonas syringae [109]). Functional analysis indicated that the PBS1 gene encodes a protein kinase which normally interacts with the Coiled Coil (CC)-NBS-LRR domain of PRS5, inhibiting the initiation of ETI [110]. Upon being challenged with Pseudomonas syringae strains, Arabidopsis PBS1 is cleaved from the PRS5-PBS1 complex by the protease activity of avrPphB, in which PRS5 is released and triggers a stronger R-mediated defense response [111,112].

**Rationale and Objectives**

Previous research showed that the citrus relative kumquat exhibited resistance characteristics such as cell death, leaf abscission and restricted bacterial growth in response to canker infection [113]. By subtractive cDNA library analysis and microarray
expression profiling, groups of genes were identified that were putatively involved in the
canker-kumquat interaction and some of these genes were homologous to known
defense-associated genes, such as transcription factors and receptors, suggesting that
canker challenge could trigger an active defense response and certain signaling events
in kumquat [113]. Preliminary comparisons between canker inoculated grapefruit and
kumquat indicated that defense-associated genes EDS1, NDR1, NPR2, NPR3, PBS1
and RAR1 were induced both earlier and higher in kumquat than in the susceptible
grapefruit, which may correlate with resistance/susceptibility to canker (Febres and
Khalaf unpublished results).

PTI, previously termed basal defense, provides one of the first levels of protection
against harmful microbial invasion and is typically triggered by the recognition of slowly
evolving molecules from microbes know as PAMPs [13]. Experimentally PTI can be
initiated by challenging plants with the synthetic 22 amino acids peptide (flg22), which is
a the conserved domain of the bacterial flagellin, a PAMP [25,27,34]. In our experiments
we used a synthetic peptide derived from Xcc (Xflg22), the causal agent of citrus
canker. The first objective of this research was to investigate whether Xflg22 triggered
PTI in citrus, and to compare defense-associated gene expression changes in response
to Xflg22 over time between susceptible grapefruit and resistant kumquat. This may
provide a better understanding of the role PTI has in the resistance/ susceptibility
response to canker.

As previously mentioned, huanglongbing (caused by Candidatus Liberibacter
asiaticus is another severe bacterial disease. Candidatus Liberibacter asiaticus is
believed not to possess flagella in citrus hosts based on the evidence from electron
microscopy observation and genome sequence information [9,114]. However, flagellin
domain-containing proteins (GI: 254040208 and GI: 254780513) have been identified in
the Candidatus Liberibacter asiaticus genome and they also contain the conserved 22
amino acid domain (Lflg22) near the N terminus with some variations. Our second
objective was to compare the response of grapefruit to Xflg22, Lflg22 and the archetype
flg22. This would provide insights into the basal defense response of grapefruit to
different PTI elicitors, which could be helpful in understanding the grapefruit
susceptibility.

A recent study of the small molecular compounds present in the vascular sap of
pathogen inoculated Arabidopsis revealed that a 9 carbon molecule named azelaic acid
was important for the resistance to virulent bacteria. In addition, the gene AZI1 was
identified to be specifically inducible by azelaic acid and mutation of AZI1 in Arabidopsis
led to compromised SAR [42]. We identified a homologous AZI1 gene in citrus and
found that its expression was induced by Xflg22 in grapefruit, suggesting that it may
have a role in the defense response and azelaic acid may also be involved in signaling.
Based on the SAR priming characteristic of azelaic acid, our third objective was to
evaluate whether exogenous application of this compound could enhance the defense
response of susceptible grapefruit. Moreover, comparing the response of defense-
associated genes to different flg22 peptides and investigating how and whether azelaic
acid affects this response could help to understand the role of these proteins in the
susceptibility of grapefruit to canker and HLB.
CHAPTER 3
MATERIALS AND METHODS

Plant Material

The plants used were greenhouse maintained 'Nagami' kumquat (*Fortunella margarita* (Lour.) Swing.) (Floyd and Associates, Dade city, FL.) and 'Duncan' grapefruit (*Citrus paradise* Macf.). All of the plants were watered daily and fertilized once a week (Peters fertilizer 20-20-20, 2.0 g/L). Horticultural oil spray was applied as needed to control mites and scales. Kumquat plants were grown in individual pots and their age were about 2 years old. Kumquat plants were pruned to the height of 70-90 cm and mature leaves were used for the experiments. Grapefruit plants were grown either individually or in groups of 2-4 plants per pot and their age were about 2-3 years. Approximately a month before the experiments, grapefruit plants were pruned to about 30-40 cm high and mature leaves from the new shoots were used for the experiments.

Flagellin22-Amino Acids Conserved Domain Peptides

The peptides used for treating the plants were obtained from GenScript USA Inc. The archetype 22-amino acid peptide (flg22, QRLSTGSRINSKADDAALQIA) was in stock from the Genscript USA Inc. and the sequence was referenced from the study by [115]. Using BLAST analysis against the NCBI protein database with the flg22 sequence as query identified a flagellin protein from *Xcc* (GI: 21242719) and two flagellin domain-containing proteins from *Candidatus Liberibacte rasiaticus* (GI: 254040208 and GI: 254780513). The conserved domains from *Xcc* (Xflg22: QRLSSSLRINSKADDAALQIA), and *Candidatus Liberibacter asiaticus* (LFgl22: DRVSSGLRVSDDAADNAAYWSIA) were then synthesized.
Challenge Kumquat and Grapefruit with Xflg22 Peptide

‘Nagami’ kumquat and ‘Duncan’ grapefruit plants were used for flg22 from Xcc (Xflg22) and control (water) treatments. The Xflg22 solution was prepared by dissolving the lyophilized peptide in distilled water to a final concentration of 10 µM. This concentration was chosen based on previous work by Zipfel et al. [25]. The Xflg22 challenge was performed by infiltrating the peptide solution into the abaxial surface of fully expanded mature leaves using a 1cc insulin syringe with a needle. Infiltration with distilled water was the control. The solution was infiltrated until half of the leaf was saturated. Subsequently, leaf samples from each treatment were collected at 0, 6, 24, 72 and 120h after the infiltration. Zero hour samples were collected right before infiltration. Three biological replicates from different plants were collected for each treatment, genotype and time point. The samples (two leaves from each plant) were immediately frozen in liquid nitrogen and stored in the freezer at -80°C; subsequently the infiltrated leaf halves were used for RNA extraction and gene expression analysis.

Azelaic Acid Pretreatment and Challenges with flg22, Lflg22 and Xflg22 Peptides

The plant material used was ‘Duncan’ grapefruit. Individual plants (a total of 24) were divided into two populations of 12 plants for each of two chemical pretreatments (azelaic acid or MES buffer). Azelaic acid (Acros Organics, NJ) was dissolved in 5mM MES (Acros Organics, NJ) buffer pH 5.6 to reach maximum solubility. The azelaic acid solution final concentration used was 1mM [42]. For the pretreatments, the 1 mM azelaic acid solution was infiltrated into the abaxial surface of leaves using a 1cc insulin syringe with a needle two days prior to the flg22 challenges and 5 mM MES buffer pH 5.6 infiltration was used as control. The 12 plants from each pretreatment were further divided into four groups of three plants as biological replicates and subsequently
challenged with a 10 µM solution of flg22, Lflg22, Xflg22 or distilled water (control). Leaf samples from each plant were collected at 0, 6, 24, 72 and 120h after the peptide or water infiltration, where 0h samples were collected immediately before any infiltration. The tissue was immediately frozen in liquid nitrogen and stored in the freezer at -80°C prior to RNA extraction and gene expression analysis.

**RNA Extraction and Purification**

For each sample, RNA was extracted using TriZol reagent following the manufacturer’s instructions (Invitrogen, CA). Each sample consisted of two different leaves from the same plant. The RNA was subsequently treated with DNase followed by a cleanup protocol using the RNeasy Plant Mini Kit (QIAGEN Sciences, MD) to eliminate any DNA and protein contamination. The concentration of RNA was determined with a NanoDrop 2000c spectrophotometer (Thermo Scientific, PA) and the RNA purity was measured as the OD_{260}/OD_{280} value in which RNA with values ≥ 1.80 were considered as clean and were subjected to cDNA synthesis.

**cDNA Synthesis**

The purified RNA (1 µg) was used for each 20 µL cDNA synthesis reaction. The 20 µL reaction mixture included 2 µL of 50 µM Random Decamers (Ambion Inc, Applied Biosystem, CA), 2 µL of 10 µM dNTPs, 2 µL of 10X First Strand Buffer (Ambion Inc, Applied Biosystem, CA), 1µL of M-MLV Reverse Transcriptase (100U/µL, Ambion Inc, Applied Biosystem, CA) and 1 µL of RNase Inhibitor (40U/µL, Ambion Inc, Applied Biosystem, CA). The remainder of the volume was completed with RNase-free water. The RNA, Random Decamers, dNTPs and water mixture was incubated at 80°C in a water bath for 2-3 minutes and placed on ice for 3 minutes before adding the First Strand Buffer, RNase Inhibitor and Reverse Transcriptase. The 20 µL reaction mixture
was then subjected to reverse transcription using the thermal cycle at 42°C for 1h and 92°C for 10 minutes (PTC-100 Programmable Thermal Controller, MJ Research Inc. Canada). The cDNA samples were stored at -20°C prior to real-time PCR.

**Gene Expression Analysis**

Gene expression levels were determined by quantitative real-time PCR using the StepOnePlus Real-Time PCR system (Applied Biosystems, CA). A Fast 96-well Reaction Plate (0.1 mL) (MicroAmp®, Applied Biosystems) was used for the reactions and each well was used to perform the 20 µL expression assay of one gene from each sample. The cDNA was diluted to a final concentration of 50 ng/µL and gene amplification was performed from 2 µL of the diluted cDNA. Each reaction mixture was composed of 2 µL of 50ng/µL cDNA, 10µL of TaqMan Fast Universal PCR Master Mix (2X) (Applied Biosystems, CA), 1 µL of TaqMan probe and primer Assay Mix (20X) (Applied Biosystems, CA) and 7 µL of RNase-free water. The Assay Mix was a combination of specific probes and forward and reverse primers for each gene. For all the assayed citrus defense genes, the final primer concentrations were 900 nM each and the final probe concentration was 250 nM. For the reference gene, 18S, the final primer concentrations were 250 nM and the final probe concentration was 150 nM per reaction.

The quantitative real-time PCR experiment was designed using the StepOne software v2.1 (Applied Biosystems, CA), in which the experiment type was set as comparative $C_T$ ($\Delta\Delta C_T$), using TaqMan Reagent and the amplification speed was set to fast. The fluorescent reporter for the tested genes was FAM and VIC for the 18S. The Quencher was NFQ-MGB for all the probes. The real-time PCR thermal cycle was 95 °C 20 for sec, followed by 95°C for 1 sec and 60°C for 20 sec. For each 96-well plate,
the amplification plots of every gene were manually checked for the correct threshold levels and proper base line starting and ending points. For the comparative \( C_T \) analysis, the 0 h samples were selected as reference samples and the calculated relative quantitation (RQ) values were exported to Microsoft Office Excel for further analysis. The RQ data were subjected to a statistical Q-test [116] to eliminate any outlier values among the replicates, and subsequently RQ means and stand errors were calculated. The statistical analysis was performed using JMP Genomic 5.0 Model fitting of standard least square means (LS Means) and Student’s t test (SAS Institute Inc. NC). All treatments for each experiment (time and type of flg22 or time, flg22 and pretreatment) were compared to each other. Mean RQ values that were statistically significant at a specific time point are indicated in the figures.
CHAPTER 4
COMPARISON OF THE RESPONSE TO XFLG22 BETWEEN CANKER SUSCEPTIBLE AND RESISTANT CITRUS TYPES

Results

Canker resistant kumquat and susceptible grapefruit were challenged with Xflg22 (water infiltration was used as the control) and samples were collected at 0, 6, 24, 72 and 120h after the treatments. For each genotype and time point the gene expression level of the following genes was determined using quantitative real-time PCR: EDS1, RAR1, SGT1, EDR1, PBS1, NDR1, EDS5, ICS1, PAL, NPR1, NPR2, NPR3, PR1, RdRP and AZI1. These genes were chosen because they are part of PTI/ETI induction (EDR1, EDS1, NDR1, PBS1, RAR1 and SGT1) (Figure 4-1), SA biosynthesis and signaling (EDS5, ICS1, PAL and AZI1) (Figure 4-2 and 4-5), PTI/ETI transcriptional regulation (NPR1, NPR2 and NPR3) (Figure 4-3) and PR genes (PR1 and RdRp) (Figure 4-4).

Among the PTI/ETI-associated genes investigated, EDS1, NDR1, RAR1 and SGT1 displayed significant elevated expression levels at 6h after inoculation compared with the water inoculated controls in kumquat (Figure 4-1A). This effect lasted for 24h (RAR1 and SGT1) or for 72 h (EDS1, NDR1) after the treatment (Figure 4-1A). In contrast, the effect of the Xflg22 in grapefruit for those same genes was different, with their expression levels not significantly different between the Xflg22 and water treatments at all the time points except 6 h (Figure 4-1B).

SA biosynthesis may be accomplished via two distinct pathways in which ICS1 and PAL catalyze the first enzymatic steps respectively. Remarkably, PAL expression was induced by Xflg22 at 6 and 24h after the treatment in kumquat but not in grapefruit.
compared with the mock inoculated controls at the same time points, even though in grapefruit PAL showed higher expression levels at 6 (water), 72 (water and XFlg22) and 120h (water and XFlg22) after the treatments compared with its expression at 0h (Figure 4-2). In contrast, ICS1 expression appeared not to be affected by XFlg22 treatment in kumquat but significantly induced in grapefruit at 24 and 72h after treatment (Figure 4-2). The SA signaling gene EDS5 did not show obvious induction by Xflg22 in either of citrus genotypes (Figure 4-2).

AZI1 is a gene regulated specifically by the defense priming signal azelaic acid, which is believed to be an essential signal for SA accumulation during the defense response in Arabidopsis [42]. In our research, AZI1 appeared to be induced by Xflg22 both in kumquat and grapefruit (Figure 4-5), although the induction happened earlier in kumquat (6h after inoculation) than in grapefruit where AZI1 was significantly induced at 72h after inoculation. The significant AZI1 upregulation in grapefruit correlated with the induction of the SA synthesis gene ICS1 but not that of PAL (Figures 4-2B and 4-5B); whereas the AZI1 upregulation in kumquat appeared to be associated with the induction of PAL (Figures 4-2A and 4-5A).

The PTI/ETI transcriptional regulation genes are shown in the Figure 4-3. Compared to the controls, expression of NPR2 was significantly higher at 6 h and 24 h in kumquat plants treated with Xflg22, and similar induction was found in NPR3 at 6, 24 and 72h after the treatment in kumquat (Figure 4-3A). NPR1 was not induced by Xflg22 compared with control, but its expression level at 6h (both water and Xflg22 treatments) was higher than 0h (Figure 4-3A). However, Xflg22 treatment in grapefruit did not trigger higher expressions of NPR1, NPR2 or NPR3 than the control treatment, even though
the induction of these genes was found in control plants at 6h in respect to 0h, which may be the result of infiltration procedure (Figure 4-3B).

PR1 is considered as the marker gene for SAR because its expression correlates with the resistance response against pathogens and SA accumulation in systemic tissues [76,77]. Even though investigating SAR was not our purpose, since the SA-mediated defense response is also an essential local defense mechanism we measured the expression of PR1 (Figure 4-4). In Xflg22 treated kumquat, PR1 expression was significantly lower than the water treated controls at 6, 24 and 72h (Figure 4-4A), which was in contrast with the PR1 expression pattern in the Xflg22 treated grapefruit where PR1 was highly upregulated at 24 and 72h after inoculation (Figure 4-4B). In addition, another PR family protein RdRP encoding gene showed significantly higher expression level than the control at 6, 24 and 72h in kumquat, whereas such induction was not found in grapefruit (Figure 4-4B).
Figure 4-1. Effect of Xflg22 on expression of EDR1, EDS1, NDR1, PBS1, RAR1 and SGT1 in ‘Nagami’ kumquat (A) and ‘Duncan’ grapefruit (B). Leaves were infiltrated with 10µM Xflg22 or distilled water. Samples were collected at 0, 6, 24, 72 and 120h after the infiltration. Gene expression was quantified by real time PCR followed by comparative C_\text{t} analysis. The vertical axis indicates the relative quantitation (RQ), where the gene expression level in each sample is compared to the reference sample (0h). The lateral axis shows the names of the biological groups including hours after inoculation and treatment, in which ‘C’ stands for control (water) and ‘X’ for Xflg22 treatment. Leaves for 0h were collected right before infiltration. The means and standard errors of three replicates are shown. An asterisk indicates a statistically significant difference between the control and treatment at the same time point.
Figure 4-2. Effect of Xflg22 on expression of EDS5, ICS1 and PAL in ‘Nagami’ kumquat (A) and ‘Duncan’ grapefruit (B). Leaves were infiltrated with 10µM Xflg22 or distilled water. Samples were collected at 0, 6, 24, 72 and 120h after the infiltration. Gene expression was quantified by real time PCR followed by comparative CT analysis. The vertical axis indicates the relative quantitation (RQ), where the gene expression level in each sample is compared to the reference sample (0h). The lateral axis shows the names of the biological groups including hours after inoculation and treatment, in which ‘C’ stands for control (water) and ‘X’ for Xflg22 treatment. Leaves for 0h were collected right before infiltration. The means and standard errors of three replicates are shown. An asterisk indicates a statistically significant difference between the control and treatment at the same time point.
Figure 4-3. Effect of Xflg22 on expression of NPR1, NPR2 and NPR3 in ‘Nagami’ kumquat (A) and ‘Duncan’ grapefruit (B). Leaves were infiltrated with 10µM Xflg22 or distilled water. Samples were collected at 0, 6, 24, 72 and 120h after the infiltration. Gene expression was quantified by real time PCR followed by comparative C_T analysis. The vertical axis indicates the relative quantitation (RQ), where the gene expression level in each sample is compared to the reference sample (0h). The lateral axis shows the names of the biological groups including hours after inoculation and treatment, in which ‘C’ stands for control (water) and ‘X’ for Xflg22 treatment. Leaves for 0h were collected right before infiltration. The means and standard errors of three replicates are shown. An asterisk indicates a statistically significant difference between the control and treatment at the same time point.
Figure 4-4. Effect of Xflg22 on expression of PR1 and RdRP in ‘Nagami’ kumquat (A) and ‘Duncan’ grapefruit (B). Leaves were infiltrated with 10µM Xflg22 or distilled water. Samples were collected at 0, 6, 24, 72 and 120h after the infiltration. Gene expression was quantified by real time PCR followed by comparative C_T analysis. The vertical axis indicates the relative quantitation (RQ), where the gene expression level in each sample is compared to the reference sample (0h). The lateral axis shows the names of the biological groups including hours after inoculation and treatment, in which ‘C’ stands for control (water) and ‘X’ for Xflg22 treatment. Leaves for 0h were collected right before infiltration. The means and standard errors of three replicates are shown. An asterisk indicates a statistically significant difference between the control and treatment at the same time point.
Figure 4-5. Effect of Xfg22 on expression of AZI1 in ‘Nagami’ kumquat (A) and ‘Duncan’ grapefruit (B). Leaves were infiltrated with 10µM Xfg22 or distilled water. Samples were collected at 0, 6, 24, 72 and 120h after the infiltration. Gene expression was quantified by real time PCR followed by comparative CT analysis. The vertical axis indicates the relative quantitation (RQ), where the gene expression level in each sample is compared to the reference sample (0h). The lateral axis shows the names of the biological groups including hours after inoculation and treatment, in which ‘C’ stands for control (water) and ‘X’ for Xfg22 treatment. Leaves for 0h were collected right before infiltration. The means and standard errors of three replicates are shown. An asterisk indicates a statistically significant difference between the control and treatment at the same time point.
Discussion

The results in this study suggest that the Xflg22 challenge triggers the expression of a set of defense-associated genes in canker resistant kumquat but not in the susceptible grapefruit. The defense-associated genes significantly induced in kumquat as a result of Xflg22 treatment were EDS1, SGT1 NPR2, NPR3, NDR1, RAR1 and PAL (Figure 4-1, 4-2 and 4-3). Among these genes, signaling regulators EDS1 and NDR1 are well known for their roles in R-mediated resistance, and there have also been reports indicating that EDS1 and NDR1 are necessary for *Arabidopsis* PTI during pathogen attack and non-host interactions with wheat powdery mildew and *Pseudomonas syringae* pv. tabaci [46,48,53,117]. Similarly, *Nicotiana benthamiana* SGT1 was found to be involved in the non-host resistance to *Pseudomonas syringae* pv. phaseolicola and *Xanthomonas axonopodis* pv. vesicatoria [62] and both RAR1 and SGT1 in soybean were necessary for PTI signaling [65]. The transcriptional alterations in flg22 treated *Arabidopsis* cell cultures and seedlings were screened using microarrays and the majority of the genes that were rapidly elicited were homologous to defense signal perception genes including NDR1, EDS1 like gene and NPR1 [118]. In our research, the induction of PTI-associated genes by Xflg22 in kumquat was in agreement with previous research results from *Arabidopsis* and other plant species, suggesting that this PAMP upregulated a similar set of PTI genes in kumquat. In contrast, grapefruit PTI signaling genes were not induced by Xflg22 treatment or the effect was too weak to be detected. Alternatively, it is also possible that there was PTI gene response but it occurred at time points beyond the 6-120h range studied here. These genes would have been either transiently induced prior to 6h after Xflg22 infiltration or late induced after 120h. However, since grapefruit is highly susceptible to
canker, the most likely scenario is that the attenuated immune response observed during the 6-120h period was indication of its inability to respond to Xflg22 and accounts for its susceptibility.

SA accumulation is correlated with pathogen-triggered defense responses in both local infection sites and systemic tissues [35,119,120]. Upon pathogen recognition, plants can initiate SA biosynthesis in order to activate defense mechanisms and the pathogen inducible SA synthesis is believed to occur via two pathways: the PAL pathway and the ICS1 pathway (Figure 2-3). There are reports from different plant-pathogen systems implying that either one of the pathways can be responsible for an increase in the SA levels during pathogen invasion [102,103,106]. In our results, Xflg22 induced significantly higher PAL expression in infiltrated kumquat but ICS1 expression did not appear to be affected, at least beyond the effect of water infiltration (Figure 4-2A). For canker resistant kumquat, the PAL catalyzed SA biosynthetic pathway might be the dominant pathway in response to Xflg22 challenge. However, it is notable that PAL is also the key enzyme in lignin biosynthesis and the lignifications of plant cells is a layer of defense during non-host incompatible interactions [106,108]. Hence the induction of kumquat’s PAL could indicate the initiation of lignin synthesis after the flg22 challenge, or perhaps PAL is involved in both SA and lignin biosynthesis during the defense response. On the other hand, ICS1 expression was the significantly induced by Xflg22 in grapefruit (Figure 4-2B). This implies that in grapefruit the ICS1 pathway might be the active SA synthetic pathway upon Xflg22 treatment. In addition, the expression of the SAR marker gene PR1 and the SA synthesis priming gene AZI1 were well coordinated with ICS1 induction (Figures 4-2B, 4-3B and 4-4B), with all three genes
upregulated at 24 and 72h after Xflg22 infiltration, suggesting that the SA regulating PR-1 expression was synthesized through the ICS1 pathway. However, RdRP as another SA inducible PR gene, was significantly induced by Xflg22 in kumquat but not in grapefruit, implying that its regulating SA may be synthesized through PAL pathway.

In Arabidopsis, NPR1 is a positive regulator of the expression of PR1 [78]. PR1 gene transcription can be activated by the binding of nuclear localized monomer NPR1 protein to the TGA transcription factor and translocation of NPR1 from its cytosol oligomer form to the nucleus is necessary for the NPR1 transactivation activity [82,84]. In the nucleus, NPR1 is also regulated by proteasome-mediated protein hydrolysis process to ensure continuous influx of active NPR1 from cytoplasm [86]. In our results, NPR1 expression was not obviously induced by Xflg22 challenge neither in kumquat nor in grapefruit (Figure 4-3), although PR1 was observed to be downregulated in kumquat and upregulated in grapefruit (Figure 4-4). The reason for the unnoticeable NPR1 induction in citrus could be the regulation of NPR1 was at the protein level instead of the mRNA level, where PR1 expression was regulated by the translocation of NPR1 to the nucleus. Alternatively, regulator(s) other than NPR1 in citrus may have a dominant role in the transcriptional regulation of PR1. Arabidopsis NPR1 has a total of five paralogs in its genome (NPR2, NPR3, NPR4, BOP1 and BOP2, Figure2-2), among which NPR3 and NPR4 form a distinctive group that have been proven to be negative regulators of PR1 expression [87]. A previous study using BLAST analysis of AtNPR1 against the citrus EST database revealed that multiple NPR1 homologous sequences exist in citrus. The evolutionary distances between these sequences and NPR1 proteins from other plant species are shown in Figure 4-6 [121]. The citrus genes CpNPR2 and CpNPR3
(from *Citrus paradisi*), which share high sequence similarity at the protein level with AtNPR3 and AtNPR4, were significantly induced by Xflg22 in kumquat whereas the expression of the genes appeared to be reduced in grapefruit (Figure 4-3). Meanwhile, the PR1 gene was significantly downregulated in kumquat treated with Xflg22 however in grapefruit this gene was induced by the same treatment (Figure 4-4). These results suggest that CpNPR2 and CpNPR3 could be involved in the negative regulation of PR1 in citrus, but the regulating roles of these two genes should be confirmed by functional analysis.

![Phylogenetic tree of NPR1-like protein sequences from different species](image)

Figure 4-6. Phylogenetic tree of NPR1-like protein sequences from different species, including citrus using the Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 268 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. At= *A. thaliana*; Cp= *C. paradisi*; Cr= Carrizo citrange (*C. sinensis* X *P. trifoliata*); Pp= *Physcomitrella patens*; Pt= *Populus trichocarpa*; Vv= *Vitis vinifera*. [121]
Previous research on the defense responses of resistant kumquat and susceptible grapefruit challenged with Xcc demonstrated that kumquat was capable of inducing the expression of defense genes much earlier and at higher levels upon canker inoculation than was the case with grapefruit and these induced genes in kumquat were believed to be associated with the observed resistance (V. Febres and A. Khalaf unpublished results). In this research, Xflg22 also triggered the response of many PTI-related genes and NPR1-like transcriptional coactivators in kumquat but not in grapefruit (Figure 4-1, 4-2, 4-3 and 4-4), suggesting that the recognition and response to Xcc flagellin was necessary for citrus plants to successfully defend themselves against the pathogenic bacterium. By comparing kumquat genes whose induction was triggered by Xflg22 with those induced by canker inoculation, it was found that most of the defense-associated genes upregulated in response to Xflg22 (EDS1, NDR1, NPR2, NPR3 and RAR1) were also even more highly induced by canker inoculation (V. Febres and A. Khalaf unpublished), suggesting that the Xflg22 initiated PTI shared similar defense signaling pathways with canker resistance response and that the flagellin triggered PTI probably contributes to the total resistance to canker observed in kumquat.
CHAPTER 5
EFFECT OF AZELAIC ACID AND DIFFERENT FLG22 PEPTIDES ON THE DEFENSE RESPONSE OF GRAPEFRUIT

Results

Grapefruit plants were pretreated with 1mM azelaic acid or 5 mM MES buffer (as a negative control) two days prior to being infiltrated with flg22 from three different organisms or water as a control. Samples were collected at 0, 6, 24, 72 and 120h after treatment. Expression levels of the following genes were determined by real-time PCR: EDS1, RAR1, SGT1, EDR1, PBS1, NDR1, EDS5, ICS1, PAL, NPR1, NPR2, NPR3, PR1, RdRP and AZI1. The results are shown from Figures 5-1 to 5-5, where the genes were divided into the groups indicated in each figure. Figures 5-1A, 5-2A, 5-3A, 5-4A and 5-5A show the gene expression in MES buffer pretreated plants, from which we could compare the effect of the different peptide treatments. Figures 5-1B, 5-2B, 5-3B, 5-4B and 5-5B show the expression of the same genes in grapefruit pretreated with azelaic acid followed by treatment with various peptides, where we could analyze the effect of azelaic acid and possibly the overlapping effects of azelaic acid and flg22 peptides.

In MES pretreated grapefruit Xflg22 did not significantly altered the expression of the majority of the defense-associated genes, given that at most time points Xflg22 treated and mock inoculated plants showed similar expression levels of EDR1, EDS1, NDR1, PBS1, RAR1 and SGT1 (Figure 5-1A), EDS5 and ICS1 (Figure 5-2A), NPR1, NPR2 and NPR3 (Figure 5-3A), PR1 and RdRP (Figure 5-4A), even though the SA biosynthesis gene PAL was induced at 120h after Xflg22 treatment (Figure 5-2A). PR1 expression was increased at 24h in all treatments, including the water control, which intimates that the increase was probably due to the infiltration procedure or some other
environment effect (Figure 5-4A). Noticeably, Xflg22 treatment elevated the expression of AZI1 in MES pretreated grapefruit at all time points and expression was significantly higher than in the control at 72h (Figure 5-5A), which was in agreement with AZI induction by Xflg22 found from the previous experiment.

In addition to investigating the effect of Xflg22, we compared the effect of the other two flagellin peptides: flg22 and Lflg22. The three types of peptides showed similar gene expression patterns as the mock inoculation did (Figures 5-1A, 5-2A, 5-3A and 5-4A), except that PAL was induced by Lflg22 and Xflg22 but not by flg22 at 120h after the treatment (Figure 5-2A), and AZI1 expression was higher in Xflg22 treated plants than the other two peptide treatments (Figure 5-5A).

In contrast to what was observed in MES pretreated grapefruit plants, azelaic acid pretreatment had an effect on the expression of some defense-associated genes. Regardless of the different flg22 treatments, the azelaic acid pretreated plants showed induction of a set of genes including EDS1, RAR1, EDR1, EDS5, NPR1, NPR2, NPR3, PR1, RdRP and AZI1 (Figures 5-1B, 5-2B, 5-3B, 5-4B and 5-5B). On the other hand, expression of PAL was reduced by azelaic acid (Figure 5-2B). However, the effect of azelaic acid appeared to be diminished by the Lflg22 challenge, as the expression levels of EDS1, RAR1, EDR1, SGT1, NPR1, NPR2, NPR3, NDR1 and AZI1 were lower than that of the control treatment at 6, 24 and 72h (Figure 5-1B, 5-2B, 5-3B, 5-4B and 5-5B) and reduced expression of SGT1, RAR1, NPR1, NPR2, NPR3, EDS1 and EDR1 caused by Lflg22 treatment was statistically significant at the time points shown in Table 5-1.
Since azelaic acid pretreatment was able to induce defense genes in all the treatments (water, flg22, Xflg22 and Lflg22), to investigate only the effect of azelaic acid, gene expression data from all the mock inoculated plants (both MES and azelaic acid pretreated) was chosen for analysis. Compared to MES pretreatment, the set of genes induced by azelaic acid infiltration alone included EDS1, RAR1, EDR1, EDS5, PAL, NPR1, NPR2, NPR3, PR1, RdRP and AZI1. It was notable that most of these genes were similarly induced in Xflg22 treated kumquat (Table 5-2). EDS1, RAR1, NPR2 and NPR3 showed significant induction at 6h after the mock inoculation and the induction of these genes lasted until 72h (Figure 5-6). As was expected, azelaic acid treatment also elevated the expression of AZI1 and the gene induction appears to last as long as 120h (Figure 5-7).
Figure 5-1. The effect of different flg22 on the expression of EDR1, EDS1, NDR1, PBS1, RAR1 and SGT1 in grapefruit pretreated with MES or azelaic acid. Duncan grapefruit leaves were infiltrated with 5mM MES buffer (A) as control or 1mM azelaic acid (B) two days prior to flg22 challenges. Using the same infiltration method, 10uM flg22 (F), Lflg22 (L), Xflg22 (X) or water as control (C) were used to challenge the pretreated plants. Leaf samples were collected at 0, 6, 24, 72 and 120h after the flg22 inoculation. Gene expression was quantified by real time PCR and comparative CT analysis. The vertical axis indicates the relative quantitation (RQ), in which gene expression level in each sample is compared to the reference sample (0h) within the same pretreatment (azelaic acid or MES). 0h samples were collected right before flg22 infiltration. The mean and standard errors of three replicates is shown.
Figure 5-2. The effect of different flg22 on the expression of EDS5, ICS1 and PAL in grapefruit pretreated with MES or azelaic acid. Duncan grapefruit leaves were infiltrated with 5mM MES buffer (A) as control or 1mM azelaic acid (B) two days prior to flg22 challenges. Using the same infiltration method, 10uM flg22 (F), Lflg22 (L), Xflg22 (X) or water as control (C) were used to challenge the pretreated plants. Leaf samples were collected at 0, 6, 24, 72 and 120h after the flg22 inoculation. Gene expression was quantified by real time PCR and comparative CT analysis. The vertical axis indicates the relative quantitation (RQ), in which gene expression level in each sample is compared to the reference sample (0h) within the same pretreatment (azelaic acid or MES). 0h samples were collected right before flg22 infiltration. The mean and standard errors of three replicates is shown.
Figure 5-3. The effect of different flg22 on the expression of NPR1, NPR2 and NPR3 in grapefruit pretreated with MES or azelaic acid. Duncan grapefruit leaves were infiltrated with 5mM MES buffer (A) as control or 1mM azelaic acid (B) two days prior to flg22 challenges. Using the same infiltration method, 10μM flg22 (F), Lflg22 (L), Xflg22 (X) or water as control (C) were used to challenge the pretreated plants. Leaf samples were collected at 0, 6, 24, 72 and 120h after the flg22 inoculation. Gene expression was quantified by real time PCR and comparative CT analysis. The vertical axis indicates the relative quantitation (RQ), in which gene expression level in each sample is compared to the reference sample (0h) within the same pretreatment (azelaic acid or MES). 0h samples were collected right before flg22 infiltration. The mean and standard errors of three replicates is shown.
Figure 5-4. The effect of different flg22 on the expression of PR1 and RdRP in grapefruit pretreated with MES or azelaic acid. Duncan grapefruit leaves were infiltrated with 5mM MES buffer (A) as control or 1mM azelaic acid (B) two days prior to flg22 challenges. Using the same infiltration method, 10μM flg22 (F), Lflg22 (L), Xflg22 (X) or water as control (C) were used to challenge the pretreated plants. Leaf samples were collected at 0, 6, 24, 72 and 120h after the flg22 inoculation. Gene expression was quantified by real time PCR and comparative CT analysis. The vertical axis indicates the relative quantitation (RQ), in which gene expression level in each sample is compared to the reference sample (0h) within the same pretreatment (azelaic acid or MES). 0h samples were collected right before flg22 infiltration. The mean and standard errors of three replicates is shown.
Figure 5-5. The effect of different flg22 on the expression of AZI1 in grapefruit pretreated with MES or azelaic acid. Duncan grapefruit leaves were infiltrated with 5mM MES buffer (A) as control or 1mM azelaic acid (B) two days prior to flg22 challenges. Using the same infiltration method, 10uM flg22 (F), Lflg22 (L), Xflg22 (X) or water as control (C) were used to challenge the pretreated plants. Leaf samples were collected at 0, 6, 24, 72 and 120h after the flg22 inoculation. Gene expression was quantified by real time PCR and comparative CT analysis. The vertical axis indicates the relative quantitation (RQ), in which gene expression level in each sample is compared to the reference sample (0h) within the same pretreatment (azelaic acid or MES). 0h samples were collected right before flg22 infiltration. The mean and standard errors of three replicates is shown.
<table>
<thead>
<tr>
<th>Grapefruit-AzA-Lfg22</th>
<th>Time Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDS1*</td>
<td>6h, 24h, 72h</td>
</tr>
<tr>
<td>RAR1*</td>
<td>6h</td>
</tr>
<tr>
<td>SGT1*</td>
<td>6h, 24h</td>
</tr>
<tr>
<td>EDR1*</td>
<td>24h</td>
</tr>
<tr>
<td>PBS1</td>
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</tr>
<tr>
<td>NDR1</td>
<td></td>
</tr>
<tr>
<td>EDS5</td>
<td></td>
</tr>
<tr>
<td>ICS1</td>
<td></td>
</tr>
<tr>
<td>PAL</td>
<td></td>
</tr>
<tr>
<td>NPR1*</td>
<td>24h</td>
</tr>
<tr>
<td>NPR2*</td>
<td>6h, 24h</td>
</tr>
<tr>
<td>NPR3*</td>
<td>6h, 72h</td>
</tr>
<tr>
<td>PR1</td>
<td></td>
</tr>
<tr>
<td>RdRP</td>
<td></td>
</tr>
<tr>
<td>AZI1</td>
<td></td>
</tr>
</tbody>
</table>

Table 5-1. Summary of the effect of Lfg22 on gene expression after azelaic acid pretreatment in grapefruit. Genes indicated in blue displayed expression levels that were suppressed by Lfg22 treatment as compared with control. An asterisk means the alteration in expression was statistically significant compared with those of the control at the time points indicated in the right column.
<table>
<thead>
<tr>
<th>grapefruit-AzA</th>
<th>kumquat-Xflg22</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDS1*</td>
<td>EDS1*</td>
</tr>
<tr>
<td>RAR1*</td>
<td>RAR1*</td>
</tr>
<tr>
<td>SGT1</td>
<td>SGT1*</td>
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<td>EDS5</td>
</tr>
<tr>
<td>ICS1</td>
<td>ICS1</td>
</tr>
<tr>
<td>PAL</td>
<td>PAL*</td>
</tr>
<tr>
<td>NPR1</td>
<td>NPR1</td>
</tr>
<tr>
<td>NPR2*</td>
<td>NPR2*</td>
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<tr>
<td>NPR3*</td>
<td>NPR3*</td>
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<tr>
<td>PR1</td>
<td>PR1*</td>
</tr>
<tr>
<td>RdRP</td>
<td>RdRP*</td>
</tr>
<tr>
<td>AZI1*</td>
<td>AZI1</td>
</tr>
</tbody>
</table>

Table 5-2. Summary of the effect of azelaic acid in grapefruit and the effects of Xflg22 in kumquat on gene induction. Genes indicated in red were upregulated by the treatment and genes in blue were suppressed. An asterisk indicates the expression alteration was statistically significant compared to the control at one or more time points. AzA stands for azelaic acid.
Figure 5-6. Effect of azelaic acid on the expression of RAR1, NPR2, NPR3 and EDS1 in grapefruit. Data was selected from Figure 5-1 and 5-3. RQ values from water infiltration treatment in grapefruit pretreated 5mM MES buffer (C) as control or 1mM azelaic acid (A) were chosen for the comparisons. An asterisk means that the expression alteration was statistically significant compared with the MES buffer pretreatment at the same time point.

Figure 5-7. Effect of azelaic acid on the expression of AZI1 in grapefruit. Data was selected from Figure 5-5. RQ values from water infiltration treatment in grapefruit pretreated 5mM MES buffer (MES) as control or 1mM azelaic acid (AzA) were chosen for the comparisons. An asterisk means that the expression alteration is statistically significant compared with the MES buffer pretreatment at the same time point.
Discussion

The results shown here indicate that neither flg22 nor Xflg22 induced the expression of defense-associated genes including PTI-associated genes (EDR1, EDS1, NDR1, RAR1 and SGT1), SA-synthesis related genes (EDS5 and ICS1) or SA induced PR genes (PR1 and RdRP) and their transcriptional regulators (NPR1, NPR2 and NPR3) in the MES pretreated grapefruit plants (Figures 5-1A, 5-2A, 5-3A and 5-4A). However, AZI1 was the exception in that it was induced by Xflg22 but not flg22 in grapefruit pretreated with MES (Figure 5-5A). A sequence comparison between flg22 and Xflg22 revealed that the two peptides had four amino acid differences (Table 5-3), suggesting that the alternations in residues could be responsible for the difference in PAMP recognition and response in grapefruit. Moreover, when the plants were pretreated with azelaic acid, it appeared that flg22 was capable of inducing AZI1 expression (Figure 5-5B), implying that the exogenous azelaic acid could complement the low AZI1 induction by flg22 and proper recognition of the residue alternations in flg22 may be key for the plants to produce endogenous azelaic acid. However, even if the Xflg22 challenge could induce AZI1 and potentially promote azelaic acid level as a defense response in grapefruit, it seemed that the induction of AZI1/azelaic acid was not enough to trigger the induction of expression of genes associated with plant defense and SA biosynthesis. We considered the concentration of 1 mM azelaic acid applied in our experiments to be high enough to induce a response [42], however it is possible that higher levels are necessary in citrus.
Table 5-3. Sequence comparisons between the 22 amino acid peptides from flagellins of archetype (flg22), Xcc (Xflg22) and Candidatus Liberibacter asiaticus (Lflg22). The amino acid differences among the three peptides are shown in red. Amino acid differences in Lfgl22 from the other two sequences are indicated in green.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>flg22</td>
<td>Q R L S T G S R I N S A K D D A A G L Q I A</td>
</tr>
<tr>
<td>Xflg22</td>
<td>Q R L S S G L R I N S A K D D A A G L A I S</td>
</tr>
<tr>
<td>Lflg22</td>
<td>D R V S S G L R V S D A A D N A A Y W S I A</td>
</tr>
</tbody>
</table>

For the huanglongbing causal agent Candidatus Liberibacter asiaticus, it is believed that this intracellular bacterial pathogen does not have a flagellar structure [9,114], even though we obtained a flagellin domain containing proteins from its genomic database. The Lflg22 peptide derived from these proteins had more amino acid differences from the sequences from the other two species (Table 5-3). Compared with the mock and the other two peptide inoculations, Lflg22 treatment showed a suppressive effect on the expressions of the majority of the defense-associated genes in grapefruit that were pretreated with azelaic acid but not in the plants pretreated with MES buffer (Figures 5-1, 5-2, 5-3, 5-4, Table 1). Genome sequencing of the bacteria reveals that Candidatus Liberibacter asiaticus has a comparatively smaller genome than other Liberibacter species and does not have type II and type III secretion systems that are necessary for pathogenicity in other bacterial species, as a result of which Candidatus Liberibacter asiaticus is considered to infect plants using an ‘avoidance strategy’ [114]. The sequence variation of Lflg22 in the flagellin domain containing protein may be a result of faster evolution of Ca. L. asiaticus and may contribute to avoiding the PAMP
recognition [114], which renders the plants more vulnerable to this pathogenic bacterium.

When comparing the effects of different flagellin peptide treatments in MES buffer pretreated plants, it was noticed that PAL was induced by Xflg22 and Lflg22 but not by flg22 and the induction event happened as late as 120h after treatment (Figure 5-2A). As discussed in the previous chapter, PAL is a key enzyme shared by the SA biosynthetic pathway and lignin biosynthetic pathway [106,108] and the induction of PAL could be an indication of initiation of either or both pathways in the response to a PAMP. Increased expression level of PAL by Xflg22 or Lflg22 suggested that this gene may be important for grapefruit recognition of pathogenic bacteria, even though the late induction could lead to insufficient defense level against the pathogens. On the other hand, in azelaic acid pretreated grapefruit, the effect of Xflg22 and Lflg22 on PAL expression was not obvious, and the average PAL expression level was lower than that of MES pretreated grapefruit (Figure 5-2B, Table 5-2), suggesting that azelaic acid may have an antagonistic role to PAL involved pathways.

Although demonstrated as an important signal for SAR priming, azelaic acid can confer both local and systemic resistance to virulent bacterial PmaDG3 in Arabidopsis when infiltrated into local leaves, and gene mutants in the SA-mediated defense pathways show compromised resistance after azelaic acid treatment [42]. Our results showed that local azelaic acid leaf infiltration alone induced a set of defense-associated genes including EDS1, RAR1, EDR1, EDS5, PAL, NPR1, NPR2, NPR3, PR1, RdRP and AZI1in grapefruit (Table 5-2). Many of these genes such as EDS1, RAR1, EDS5, PAL, NPR1, PR1, RdRP and AZI1are associated with the SA-mediated defense
signaling, suggesting that azelaic acid application induced a similar local defense response in citrus as it did in *Arabidopsis*. Additionally, comparison between the azelaic acid alone infiltrated grapefruit and Xflg22 challenged kumquat indicated that azelaic acid was capable of triggering the expressions of a set of grapefruit genes that seemed necessary for the basal defense in kumquat (Table 5-2), and these genes had also been shown to be highly induced by *Xcc* in kumquat (V. Febres and A. Khalaf unpublished results), which was further evidence for the mediation of defense by azelaic acid in grapefruit. However, whether the azelaic acid mediated defense response would be sufficient to protect grapefruit plants against pathogen invasion still needs to be confirmed by pathogen challenge assays.

In *Arabidopsis*, AZI1 induction was confirmed from 3h-48h after leaf infiltration with 1mM azelaic acid [42]. In our research, due to the different experimental design in which all the samples were collected after the azelaic acid/MES pretreatment (two days before), the time points when the samples were used for gene expression assays were actually 48 hours later. Hence the AZI1 induction by the azelaic acid treatment ranges from 0-120h in grapefruit is equivalent to 48h-168h (Figure 5-2) in the *Arabidopsis* experiment, indicating that azelaic acid can have an effect on the AZI1 expression for at least more than five days. Homologous to a lipid transfer protein family, AZI1 is believed to be the regulator or transporter of SAR priming signal in *Arabidopsis* [42]. In grapefruit, the induction of AZI1 by azelaic acid implies that AZI1 can have a similar role during the SAR as in *Arabidopsis* in mediating azelaic acid primed resistance.
CHAPTER 6
CONCLUSIONS

The results presented here show that: 1) Xflg22 induced a number of PTI-associated genes (EDS1, SGT1, RAR1 and NDR1) and transcriptional activators (NPR2 and NPR3) in canker resistant kumquat but not in susceptible grapefruit, suggesting that PTI has a role in canker resistance; 2) Grapefruit did not respond differently to flg22, Xflg22 or Lflg22, suggesting that is not capable of sensing this peptide, contributing to its susceptibility; 3) The application of Azelaic acid (1 mM) alone induced a defense response in susceptible grapefruit that was comparable to Xflg22 induced immunity in resistant kumquat. However, when grapefruit plants were pretreated with azelaic acid, Lflg22 showed a suppressive effect on the expression of defense-associated genes whereas flg22 and Xflg22 did not; 4) AZI1 was induced by Xflg22 and azelaic acid in grapefruit, suggesting it is involved in the defense response of citrus as is the case in model systems; and 5) It remains to be determined whether azelaic acid can be used effectively and practically in the control of citrus canker.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Qingchun Shi was born in Baoding, Hebei province, China. He graduated from Agricultural University of Hebei with a Bachelor of Agronomy, majoring in horticulture. During his undergraduate study, he worked in the project ‘Research on Ya Pear and its Eco-geological and Chemical Environment’ and finished his thesis titled in ‘Dynamics of Soil Nutrients in Pear Orchard in Spring’. Due to his excellent performance in study and research, he was selected as graduate student candidate without an entrance exam in Agricultural University of Hebei, majoring in pomology. During his graduate study, he was involved in the projects ‘Technology and Extension of Improvement of Pear fruit quality’, concentrating on tissue culture and Agrobacterium-mediated transformation of insect-resistant gene in pear cultivars. In parallel, he completed his master’s thesis research ‘Effects of Salicylic Acid on Respiratory Pathway of Postharvest Huang-guan Pear’ and obtained the Master's degree in 2009. In the fall of 2009, he was admitted to the plant molecular and cellular biology (PMCB) program in University of Florida. During the two year’s graduate study in PMCB, he did the master’s research on ‘Flg22-Triggered Immunity and the Effect of Azelaic Acid on the Defense Response in Citrus’.