MODULATION OF INNATE IMMUNITY IN ARABIDOPSIS

by

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Abstract

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Plants possess a highly sophisticated defense system to fight against pathogen infection. One of the components of this system, stomatal immunity, was studied here. Stomata, formed by a pair of epidermal guard cells, are tiny apertures on leaf surfaces that regulate exchange of gases and water loss in the plant. Apart from this function, stomata actively close in response to pathogens, thereby preventing entry of the pathogen in the plant. This constitutes stomatal immunity. However, in this study it was seen that stomatal immunity in *Arabidopsis thaliana* is suppressed when infection occurs in high relative humidity (RH). Now, opening and closing of stomata relies on a complex network of signaling in guard cells. Regulation of some components of this signaling was assessed in high relative humidity, to explain suppression of stomatal immunity and higher incidence of disease in high RH. Arabidopsis immunity was also studied when the plant encounters a human pathogen. It was observed that infection with the enteric pathogen *Salmonella enterica* compromises stomatal immunity and some components of apoplastic defense in Arabidopsis. These studies will be beneficial in the advancement of the fundamental knowledge of early events in plant-pathogen interactions, thus leading to solutions for reduction in occurrence of plant diseases and food-borne illnesses.

Table of Contents

List of Illustrations

List of Tables

Chapter 1

Introduction to Plant-Pathogen Interactions

As of this month, April 2014, the human world population is reported to be 7.15 billion and growing (United States Census Bureau). The major concern with increasing population is increase in demand for food supply. Food security is one of the most pressing issues in international politics, and availability, access, and utilization of food determine world food security. With increasing population, climate change, and increased competition for land, water, and energy, the world faces a major challenge of food security with whatever natural resources currently available, in an environmentally sustainable way. A major constraint to food production is crop losses due to high occurrence of plant diseases. Different types of bacteria, fungi, nematodes, and viruses can act as plant pathogens. A pathogen can reduce the yield of the food product obtained from the crop or kill the entire plant. For example, vascular wilts can generally kill the entire plant. Frequently, the pathogen may not kill the entire crop but can reduce the yield and quality of the produce. For example, bacterial spots on fruits indicate infection and may not be consumed, but the plant may be healthy. Such food products are not preferred and mostly go waste. Crop losses lead to heavy financial losses. It is estimated that in the United States, crops worth $9.1 billion are lost to diseases, $7.7 billion to insects, and $6.2 billion to weeds (Agrios, 2005). Significant progress has been made to understand and control plant diseases, yet losses due to wheat stem rust, rice blast, witchweed, Asian soybean rust, cassava brown streak virus are witnessed today as well. This indicates that we still lack relevant information about plant-pathogen interactions and research in plant pathology is the only way we can find ways to curb plant diseases.

Plant-pathogen interaction involves several events before either a disease is manifested or the infection is cleared. Plants are equipped with a complicated network of

innate immune system to ward off pathogens. Jones and Dangl (2006) proposed an elegant model for interaction of a plant pathogen with the plant immune system. Plants are able to mount a generalized step one response towards conserved pathogen molecules, which are mostly extracellular, or modified/degraded plant products. These molecules, called pathogen or damage associated molecular patterns (PAMP/DAMP) are recognized by a diverse set of plant receptors called pattern-recognition receptors (PRRs) and this signal is passed intracellularly, which launches an army of defense molecules to stop the invasion of the pathogens. This immunity is called pathogen-triggered immunity (PTI). Successful virulent pathogens are able to defeat this army by their own set of artillery (virulence factors, type three secretion system effectors, phytotoxins) and cause disease in the host plant. In incompatible interactions, the host plant already has pre-evolved molecules (R proteins) that recognize these effectors and cause a defense response targeted to the specific pathogen. This specific response is called effector-triggered immunity (ETI), which results in resistance to disease. However, absence of specific R proteins, as in some non-host plants, leads to manifestation of disease. Hence, the winner, whether plant or the pathogen, in this arms-race of disease determines the outcome of the initial infection.

1.1 Arabidopsis is an Excellent Model Plant for Studying Plant-Pathogen Interactions

Usage of *Arabidopsis thaliana* in plant pathology is commonplace. Arabidopsis is a very powerful tool in modern plant research because of its outstanding genetic and genomic resources. It has a very small genome of 157 Mbp (Bennett et al. 2003). Its entire genome has been sequenced (The Arabidopsis Genome Initiative, 2000), and genetic and genomic resources about this model plant are available at The Arabidopsis Information Resource (TAIR, CA; www.arabidopsis.org, ARBC, OH). Moreover, T-DNA knock-out lines

are available to the public. This model organism has been extensively used for understanding molecular mechanisms underlying pathogen interaction with plants.

1.2 Virulent Pathogens *Pseudomonas syringae* and *Salmonella enterica* can be used to Study Plant-Bacterial Interactions

The model pathosystem of Arabidopsis-*Pseudomonas syringae* was used here to study plant-pathogen interactions. *P. syringae* is an economically important plant pathogen infecting a wide range of plants. There are several serovars of the bacterium, which cause disease in specific hosts. The strain used in my study is *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), that infects tomato and Arabidopsis (Whalen, 1991; Cuppels, 1986). *P. syringae* infects mostly aerial parts of the plants, and leads a hemibiotrophic lifestyle. Its lifecycle can be divided into two distinct stages, an epiphytic phase on the surface of plant, and the subsequent endophytic phase in the apoplastic (intercellular) space after penetration in the plant tissue. *Pst* DC3000 is a poor epiphyte and requires transition to the endophytic phase quickly in order to cause successful infection (Boureau et al. 2002).

Salmonella enterica is a Gram-negative bacterial pathogen that is capable of infecting humans and other animals. *Salmonella enterica* serovars can be divided into two groups – typhoidal and non-typhoidal bacteria. *S.enterica* serovar Typhimurium that I used for my research is a non-typhoidal bacterium that causes gastroenteritis, abdominal pain, vomiting, and inflammatory diarrhoea in several animals including humans. The route of infection is usually by consumption of contaminated food or water; however these bacteria can also be acquired from exposure to pet reptiles and amphibians that carry these bacteria as natural flora (Haraga et al. 2008). Along with plant pathogens, plant commensals, and plant beneficial bacteria, human pathogens are now seen to be commonly associated with plants leading to food borne-illnesses (Scallan et al. 2011). In my study, the interaction of

S. enterica serovar Typhimurium SL1344 with Arabidopsis was examined. Plants are not natural hosts of human enteric pathogen, but may act as a vector or reservoir for human pathogens. Hence, studying plant responses to human pathogens like *S. enterica* is crucial in understanding the role of plants in the lifecycle of enteric human pathogens.

1.3 Pathogen-Triggered Immunity (PTI) Consists of Several Responses

PTI is usually the first line of defense against any infection. Presence of conserved molecular patterns, PAMPs, on most microorganisms is the trigger for PTI to be activated. Some examples of PAMPs are lipopolysaccharide (LPS), chitin, flagellin (Zeng et al. 2010), and elongation factor (EF-Tu) (Kunze et al. 2004). Recognition of these PAMPs by PRRs leads to several defense responses which can be categorized based on the time of response. Early responses occur within seconds to minutes of recognition and include ion fluxes, oxidative burst. Intermediate responses, occurring within minutes to hours include stomatal closure, ethylene production, mitogen-activated protein kinase (MAPK) signaling, and transcriptional reprogramming. Late responses, occurring from hours to days, involve callose deposition, salicylic acid accumulation, and defense gene expression (Zipfel and Robatzek, 2010).

1.4 Plant Stomata: Immunity Gates at the Leaf Surface

Apart from providing a habitat for several pathogenic as well as non-pathogenic microorganisms, the leaf surface can be a very harsh environment. Nonetheless, bacteria, the most abundant organism on the leaf surface (Lindow and Brandl 2003) with density of 10^6-10^7 cells/cm^2 of leaf (Andrew and Harris, 2000), have evolved mechanisms to either avoid or tolerate those stresses. In contrast, the leaf interior is a favorable environment full of photosynthates that can be exploited by bacteria. However, the transition from epiphytic

to endophytic lifestyle is not easy and the entry of pathogen into the plant tissue is crucial to cause a successful infection. The plant surface has its own physical barriers, such as the cuticle and the epidermis, against bacterial invasions. As bacteria cannot directly penetrate through these barriers, they have to rely on natural entry points located on the plant surface, out of which, stomata represent one of the most important routes for bacterial entry (Melotto et al. 2006). Plant stomata, tiny pores present on the phyllosphere of all plants, are required for gas exchange and transpiration to maintain the normal physiology of the plant. Each stoma is surrounded by a pair of specialized epidermal cells called as guard cells. Changes in the turgor pressure of the guard cell cause the opening and closing of the stomatal pore (Schroeder et al. 2001). Stomata are also natural entry points for pathogens to gain entry into the plant interior and important for the first step of infection. Previously, it was assumed that entry of bacteria into the plant is a passive process where it was dependent mostly on the chance of the bacteria encountering stomata. However, the penetration of bacteria in the leaf tissue via stomata is not just a simple act of swimming into the leaf through passive openings, but it is much more complex and dynamic (Melotto et al. 2006). Stomata act as a part of the plant innate immunity by closure of the aperture in response to recognition of bacterial pathogen on the leaf surface (Melotto et al. 2006). This constitutes the phenomenon of stomatal immunity.

Bacterium-induced stomatal closure is an important part of plant immune defenses and requires the FLS2 receptor, production of nitric oxide (NO), salicylic acid (SA) homeostasis, abscisic acid (ABA) signaling components, such as the guard-cell-specific OST1 kinase (Melotto et al. 2006), K+ channel regulation via heterotrimeric G-Protein (Zhang et al. 2008), and Mitogen-Activated Protein Kinase3 (MPK3; Gudesblat et al. 2009). However, certain bacterial pathogens can successfully cause disease by overcoming this stomatal defense like *Xanthomonas campestris* pv. *campestris* (Gudesblat et al. 2009),

Pseudomonas syringae pv. *syringae* B728a (Schellenberg et al. 2010), *P. syringae* pv. *tabaci* (Melotto et al. 2006), *P. syringae* pvs. *tomato* and *maculicola* (Melotto et al. 2006). Specifically, the plant pathogen *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) produces a phytotoxin called coronatine (COR) for this purpose.

1.5 Coronatine Acts by Modulating Plant Hormone Signaling

Coronatine (COR) is a nonhost-specific phytotoxin with a significant role in bacterial infection in plants, importantly promoting pathogen entry though stomata by suppression of stomatal immunity (Melotto et al. 2006). This phytotoxin is produced by several pathovars of *P. syringae* including *tomato*, *maculicola, glycinea,* and *atropurpurea*. It modifies the plant's physiology and induces chlorosis in infected leaves (Bender et al. 1999; Brooks et al. 2004; Mittal and Davis 1995). COR-producing strains of *P. syringae* have been found to be more aggressive than its derivative COR-defective mutants (Brooks et al. 2004; Ishiga et al. 2009). COR is a structural and functional mimic of the plant hormone jasmonoyl isoleucine (JA-Ile; Katsir et al. 2008; Melotto et al. 2008; Zhao et al. 2003). JA-Ile is a lipid derived plant hormone with regulatory functions in vegetative and reproductive growth, defense responses against abiotic stresses such as ultraviolet light and ozone, insect herbivory, and necrotrophic pathogens (Katsir et al. 2008). COR activates jasmonic acid (JA) signaling, induces JA-responsive genes in Arabidopsis, and contributes to disease development by antagonizing salicylic acid (SA) signaling, a plant hormone actively involved in plant defense against *P. syringae* (Glazebrook et al. 2003; Uppalapati et al. 2007). Specifically, in JA pathway, COR, mimicking JA-Ile, binds to COI1 protein that releases JA signaling from repression (Fig. 1.1). *COI1* gene encodes for an F-box protein (Xie et al. 1998) that is associated with SCF, a type of E3 ubiquitin ligase consisting of SKP1, CULLIN1, and F-box proteins (Devoto et al. 2002; Xu et al. 2002) that

tags proteins for degradation through the 26S proteasome, in this case the repressors of JA response signaling, called as JAZ (JASMONATE ZIM-DOMAIN) proteins. This leads to the expression of JA-responsive genes and activation of JA-mediated responses (Fig. 1.1). The mode of action of COR and JA in the plant cell has been the subject of intensive research (Melotto et al. 2008; Baker et al. 2010; Pauwels and Goossens, 2011).

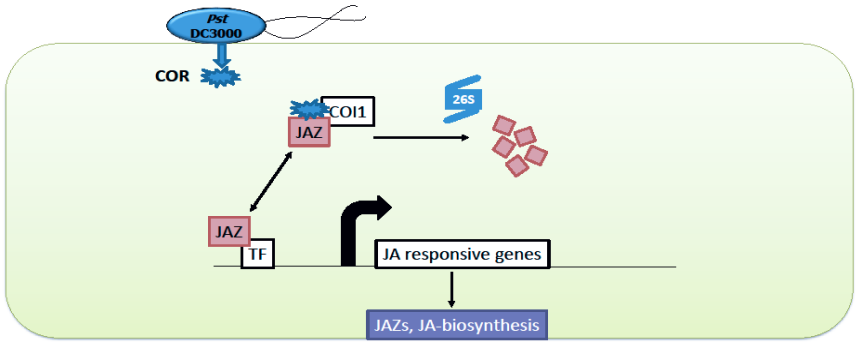

Figure 1.1 Coronatine (COR) interferes with the JA pathway in Arabidopsis. COR secreted by *Pst* DC3000 binds to the F-box protein, COI1, which leads to degradation of JAZ repressor proteins through the 26S proteasome. This releases the repression on transcription of JA-responsive genes like *JAZs* and JA biosynthetic genes.

1.6 Environmental Factors are of Crucial Importance in Studying Plant-Pathogen Interactions

The disease triangle is a textbook understanding in plant pathology. For occurrence of a plant disease, three factors have to be fulfilled – virulent pathogen, susceptible host, and favorable environmental conditions. The length of each side of the triangle corresponds to the sum total of characteristics of that factor that favor disease and the area of the triangle is related to the severity of disease (Agrios, 2005). Plant stomata are highly influenced by environmental conditions, like light, humidity, carbon dioxide levels, and temperature. So, the penetration of a pathogen through stomata should be studied in the varied environmental conditions that influence opening and closing of

stomata; *i.e.,* guard cell movement in the presence of both biotic and abiotic factors should be studied simultaneously. Stomatal aperture is under the control of guard cells. Intricate guard cell signaling pathways are involved in opening and closing of stomata in response to biotic and abiotic stresses, including ion fluxes, sugar transport, cytoskeleton rearrangement, hormone signaling, and gene expression (Schroeder et al. 2001; Montillet and Hirt, 2013). Hence, regulation of these cellular events during pathogen infection in different environmental conditions must be studied to predict the outcome of the plant-pathogen interaction and possibly develop solutions to avoid occurrence of disease in field.

1.7 Studying Interaction of Human Pathogens with Plants is an Important Aspect of Food Safety

It is estimated that 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases in the US (CDC, 2011). The number of serious cases leading to death has been increasing and outbreaks associated with fresh produce have emerged as an important public health concern. In particular, enterohemorrhagic *Escherichia coli* and *S. enterica* appear to be the most common causal agents of food poisoning associated with the consumption of fresh leafy vegetables. *Salmonella enterica* infection causes diarrhea, fever, and abdominal cramps. The enterohemorrhagic *E. coli* O157:H7 causes bloody diarrhea and hemolytic uremic syndrome. Both of these human pathogens are not known to be plant pathogens; nonetheless they can survive on and/or penetrate into plant tissues, causing serious food borne disease outbreaks. The presence of human pathogens like *Salmonella*, *Staphylococcus*, *and E. coli* in crop plants has been reported several times in the past (Tyler and Triplett, 2008). However, with increasing foodborne illness outbreaks, only recently have there been studies demonstrating active survival mechanisms by some of

these human pathogens inside and on plants (Barak and Schroeder, 2012; Deering et al. 2011).

1.8 Research Goal

The main goal of this research was to study the changes in the Arabidopsis defense system in the presence of different biotic and abiotic factors. In Chapter 2, two factors that suppress immunity, coronatine and high relative humidity (RH) were studied. Specifically, defense regulation in Arabidopsis leaves was assessed when plants were exposed to high humidity conditions. Although observation of high disease occurrence in the rainy season is common, the mechanisms that influence the plant to be unable to defend itself have not been studied well. My research focused on regulation of stomatal immunity when exposed to high relative humidity (RH) conditions. In addition, epiphytic virulence of *Pst* DC3000 was also studied with respect to coronatine production. This is important for understanding how this bacterium transitions from epiphytic to an endophytic lifestyle. Chapter 3 focusses on modulation of Arabidopsis immunity in the presence of a human pathogen, *Salmonella enterica* serovar Typhimurium SL1344. Inactivation or modulation of plant defenses by a human pathogen is a relatively new field of study. Evidences of occurrence, survival, and behavior of human pathogens like *S. enterica, E. coli* O157:H7, *Listeria,* and *Norovirus,* have been documented, but there have been very few studies describing plant responses to human pathogens. Human pathogens may use plants as vectors or temporary reservoirs to be able to infect the animal host (cattle, poultry) and continue the lifecycle (Barak and Schoreder, 2012). Disrupting the step of survival on or in the plant can be a good strategy for prevention of food-borne outbreaks. To do this, however, it is important to understand why plants are unable to clear human pathogen infection. Hence, plant immunity in the presence of one of these human pathogens, *Salmonella enterica* serovar Typhimurium SL1344 was assessed.

Chapter 2

Factors That Suppress Stomatal Immunity

2.1 Abstract

It has long been observed that environmental conditions play crucial roles in modulating immunity and disease in plants and animals. For instance, many bacterial plant disease outbreaks occur after periods of high humidity and rain. A critical step in bacterial infection is entry into the plant interior through wounds or natural openings, such as stomata. Recent studies have shown that stomatal closure is an integral part of the plant immune response to reduce pathogen invasion. In this study, it was discovered that high humidity can effectively compromise stomatal immunity in both common bean and Arabidopsis against *Pseudomonas syringae*. The molecular components involved are up-regulation of the jasmonic acid pathway, downstream of or independent of COI1, and down-regulation of the salicylic acid pathway. In addition to this, it was also observed that coronatine biosynthetic genes are activated on the leaf surface indicating possible recognition of the leaf environment by *Pst* DC3000 in preparation for the virulent, endophytic phase of its life cycle.

2.2 Introduction

The plant phyllosphere is one of the most diverse niches for microbe inhabitation. Numerous bacteria can survive and proliferate on the surface of the plant without causing any harm (Lindow and Brandl, 2003). However, for a bacterial pathogen to cause a successful infection, it must penetrate through the plant epidermis and be able to survive and proliferate inside of the plant. The mode and mechanism of penetration into the plant tissue is a critical step for infection, especially for bacterial pathogens that rely on natural openings and accidental wounds on the plant surface to colonize internal tissues (Misas-

Villamil et al. 2013). Stomata are such openings, providing a main route through which foliar bacterial pathogens transition from epiphytic to endophytic lifestyles. It has been shown that plants are able to respond to human and plant bacterial pathogens by actively closing the stomatal pore (Gudesblat et al. 2009; Hettenhausen et al. 2012; McDonald and Cahill, 1999; Melotto et al. 2006; Zhang et al. 2010; Roy et al. 2013). Several lines of evidence point to the complexity of this response and show that stomatal closure is an integral basal plant defense mechanism to restrict the invasion of pathogenic bacteria into plant tissues (Gudesblat et al. 2009; Melotto et al. 2006; Ali et al. 2007; Zhang et al. 2008). However, *P. syringae* pv. *tomato* (*Pst*) DC3000 uses the phytotoxin coronatine (COR) to overcome stomatal immunity and gain entry inside the leaf tissue to cause disease. For this, COR binds to the F-box protein, COI1, and this releases JA signaling from repression. This leads to the expression of JA-responsive genes and activation of JA-mediated responses.

One important aspect of studying the action of COR is to know the location and timing of COR production. COR is a virulence factor and hence its synthesis might be induced in the bacterium in some way by recognition of the leaf environment. Knowing that COR is able to re-open stomata, it can be hypothesized that COR induction might occur before bacterial penetration in the leaf, *i.e.,* during the epiphytic stage of the pathogen. This hypothesis was tested in this study in regular conditions as well as in dark environment, when stomata in most land plants are closed. It would be of utmost importance to produce COR, for infection at night.

Similar to light conditions, another environmental condition that influences stomatal movement is relative humidity (RH). Low humidity signals the guard cell to close stomata to prevent any water loss from transpiration. However, in natural conditions, plants are exposed to both biotic and abiotic stresses. Biotic stress would induce stomatal closure

while high humidity would induce stomatal opening. Hence, guard cells, that form stomata, need to prioritize their response to simultaneous occurrence of these stresses. It is a common observation that severe outbreaks of bacterial disease in the field are often associated with periods of heavy rain or high air humidity (Goode and Sasser, 1980). Interestingly, to ensure infection in the laboratory, researchers commonly expose plants to very high humidity for an extended period after surface inoculation. Extensive disease in high humid conditions can be attributed to several factors including higher multiplication rates of the pathogen and decreased host immunity. So in this study, modulation of plant immunity under high humidity and bacterial infection was studied. Here, it is hypothesized that under high RH, stomatal defenses would be compromised allowing more bacteria to enter the leaf tissue to promote infection, which can partly explain severe plant diseases occurring during rainy season. It is also important to determine the genetic basis for such occurrence. It is well known that the fully virulent COR-producing *Pst* DC3000 activates JA response pathway in a COR-dependent manner, which contributes to suppression of stomatal and apoplastic defenses and promotion of disease development. Therefore, involvement of JA pathway in high RH conditions was studied. Involvement of salicylic acid (SA) signaling was also checked as often JA and SA signaling events occur in an antagonistic manner. Moreover, SA signaling plays a positive role in stomatal immunity and apoplastic defenses against *Pst* DC3000. Hence, in this study, the effect of high relative humidity (RH) on stomatal immunity, the genetic basis for regulation of stomatal immunity by high relative humidity, and the timing and location of COR production, were studied.

2.3 Materials and Methods

2.3.1 Plant Material and Growth Conditions

Arabidopsis thaliana (L. Heyhn.) ecotype Columbia (Col-0, ABRC stock CS60000) seeds were sown in a 1:1:1 v:v:v mixture of growing medium (Redi-earth plug and seedling mix, Sun Gro), fine vermiculite, and perlite (Hummert International, Earth City, MO) and grown in controlled environmental chambers at 22^0C, 60±5% relative humidity (RH), and a 12-h photoperiod under light intensity of 100 $\mu mol.m^{-2}.s^{-1}$. Four- to five-week old plants were used for all experiments. Seeds of the *Phaseolus vulgaris* (L.) cultivar Beluga (Kelly et al. 1999) were surface sterilized with 50% bleach (Ultra Clorox® Germicidal Bleach, VWR, West Chester, PA) for 4 min, sown onto Jiffy peat pots (Hummert International), maintained under 16 h photoperiod at 25^0C at 60±5% RH, and light intensity of 140 $\mu mol.m^{-2}.s^{-1}$. Eight- to ten-day old seedlings with fully expanded primary leaves were used for the experiments. *coi1-1* mutant plants (Col-0 background) were maintained as heterozygotes and were screened on 25 µM methyl-JA-containing Murashige & Skoog medium with MES buffer and vitamins (RPI Corporation, Prospect, IL) supplemented with 30 $g.l^{-1}$ sucrose for root growth sensitivity assay. Homozygotes with extended root lengths were chosen for experiments and later confirmed for male sterility (Kloek et al. 2001).

2.3.2 Bacterial Strains and Growth Conditions

Bacterial cells were grown in low-salt Luria-Bertani medium (Katagiri et al. 2002) at 30^0C for all experiments. Medium was supplemented with the appropriated antibiotic: 100 $\mu g.ml^{-1}$ rifampicin (*Pst* strains), 50 $\mu g.ml^{-1}$ kanamycin (*Pst* DC3118, *Pst* DC3000 *hrpA*$^-$), 25 $\mu g.ml^{-1}$ chloramphenicol (*Pst* DC3000 pHW01), and 10 $\mu g.ml^{-1}$ tetracycline (*Pss* B728a syringolin A-defective mutant *syl*$^-$).

2.3.3 Stomatal Assay

For experiments to assess the effect of RH on stomatal immunity, plants were acclimated under varying RH of 60±5% and >95% for 12 h. Highly humid conditions were obtained by keeping well-watered plants covered with plastic domes in controlled environmental chambers. The level of humidity was monitored with a digital hygrometer (Traceable®, VWR). Plants from each RH condition were dip-inoculated in the morning (3-4 h after lights were turned on) as described below and leaves were collected over time for stomatal aperture measurements.

Stomatal assays with intact leaves were performed as previously described (Melotto et al. 2006; Chitrakar and Melotto 2010), except that intact leaves were directly imaged without propidium iodide staining. Stomatal aperture width was measured with a Nikon Eclipse 80i fluorescent microscope equipped with DIC and long distance objectives (Nikon Corporations, Shinagawa-ku, Tokyo, Japan) to avoid the use of a cover slip. All experiments were completed by 2 pm.

2.3.4 Bacterial Pathogenesis Assay

To assess the population of bacteria inside the leaf apoplast, bacterial pathogenesis assay was carried out. Plants were dip-inoculated with 1 x 10^8 CFU.ml^{-1} bacterial suspension containing 0.02% Silwet. Pathogenesis assays were performed as previously described (Katagiri 2002; Sabaratnam and Beattie, 2003), except that inoculated bean seedlings were kept under the same conditions used for growth.

For experiments with varying levels of RH, plants were acclimated under 60±5% or >95% RH, at 25^0C for 12 h. Highly humid conditions were obtained by keeping well-watered plants covered with plastic domes in controlled environmental chambers. The level

of humidity was monitored with a digital hygrometer (Traceable®, VWR). Immediately following infection, plants were incubated under the same RH for 3 days.

2.3.5 Guard Cell Morphology Studies

For comparison between stomata from different genotypes, leaves from the plants were imaged 3-4 hours after lights were turned on to observe stomata in untreated conditions. From each plant, three leaves were imaged with a Nikon Eclipse 80i fluorescent microscope equipped with DIC and long distance objectives (Nikon Corporations, Shinagawa-ku, Tokyo, Japan). Different factors like stomatal density (average number of stomata), stomatal aperture, size of guard cell pair, length and width of the stomatal complex, and size of the stomatal complex were measured using NIS software (Nikon Corporations, Shinagawa-ku, Tokyo, Japan).

2.3.6 Guard Cell Extraction

Four to five week old Col-0 plants were used for all guard cell extractions. At required time points, 50-75 leaves were harvested and blended in water containing transcription inhibitors, cordycepin (0.01%) and actinomycin D (0.0033%) to avoid gene expression changes due to mechanical damage. Guard cells were extracted according to Obulareddy et al. (2013) and cells were flash frozen for RNA extraction.

2.3.7 Gene Expression Analysis by Real Time PCR

Arabidopsis plants were acclimated under 60±5% RH at 25^0C for 12 h. To check the effect of high humidity on JA-responsive gene expression, plants were covered with a plastic humidity dome covered with a fine mist on the inside so that a 95±5% RH was immediately reached. Plants were not moved or disturbed during changes of RH. Plants

from different pots were used to collect leaf tissue at different time points to avoid touch or movement induction of genes. To assess the expression of SA-responsive genes, plants were dip-inoculated with *Pst* DC3118 suspension or mock inoculated and then distributed in two different humidity conditions, 60±5% and 95±5% RH. From all experiments, leaf tissue was collected at different time points and flash frozen or used for guard cell extraction and then cells were flash frozen tissue for RNA extraction.

Total RNA was extracted from whole leaves or guard cells using RNeasy Plant Mini kit including the in-column DNA digestion option (Qiagen, Valencia, CA) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL). Total RNA (1 µg) was synthesized into cDNA using the Takara RNA PCR kit (AMV) (Clontech, Montain View, CA). Quantitative PCR (qPCR) reaction (20 µL) was performed with 10 µL of iTaq Fast SYBR Green Supermix (BioRad, Hercules, CA), 2 µL of cDNA template from the reverse transcriptase reaction described above, and 200 nM of reverse and forward gene-specific primers. Reactions were carried out in an Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA) using the following cycling parameter: 1 cycle 95^0C for 5 min and 40 cycles of 95^0C for 10 sec and 58^0C for 30 sec. Gene expression levels were normalized based on the expression of the housekeeping gene, actin, *ACT8* and fold change expression relative to the control was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). Two biological replicates and three technical replicates were performed.

Gene-specific primer sets that span an intron region were designed using the primer quest software from IDT-SciTools (http://www.idtdna.com/Primerquest/Home/Index) for qPCR analysis. To assess reaction efficiencies, standard curves were created using a five-fold serial dilution of the cDNA pool. A linear regression between the amount of cDNA template and the cycle threshold (C_T)

value was calculated to obtain a correlation coefficient (R^2) >0.97. The PCR efficiency was determined according to Schmittgen and Livak (2008). All gene-specific primers are described in the Table 1.

2.3.8 cma Promoter Activity

To assess coronatine biosynthesis gene induction on the leaf surface, a reporter gene assay was employed. The abaxial side of intact Arabidopsis leaves was placed in contact with *Pst* DC3000 (pHW01) suspension on a microscopic slide and incubated at 22^0C in light or dark. *Pst* DC3000 (pHW01) contained fusion of coronatine biosynthesis promoter *cma* with *egfp* to measure transcriptional activity for coronatine biosynthesis (Weingart et al. 2004). The petioles of the leaves were not in contact with the bacteria to prevent any leaf extracts causing gene induction (Fig. 2.1). The leaf surface was imaged under a Nikon Eclipse 80i fluorescent microscope to check for the timing of GFP expression driven by the *cma* promoter.

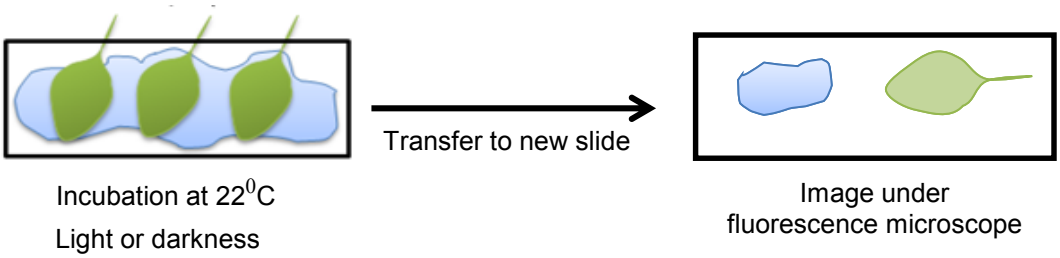

Figure 2.1 Experimental set up for monitoring *cma* promoter activity. Abaxial side of intact Arabidopsis leaves was kept in contact with *Pst* DC3000 (pHW01) bacterial suspension of 1 x 10^8 CFU.ml^{-1} on a microscope slide and incubated in constant light (80-90 µmol.m^{-2}.s^{-1}) or constant darkness. Leaf petioles were never in contact with the bacterial suspension. At different time points, the abaxial surface of the leaf was imaged under fluorescent microscope in search for fluorescent bacterial cells. Additionally, 5 µL of the bacterial inoculum in contact with the leaf was also imaged.

2.3.9 Dislodgement of Bacteria from Leaf Surface and Epiphytic Bacterial Gene Expression

To infect Arabidopsis leaf surface, *Pst* DC3000 with starting O.D of 0.8, was diluted to 0.4 and sprayed with fine mist on Col-0 leaves. After 6 h, 100 leaves were harvested to dislodge bacterial cells. For collecting these cells, leaves were shaken in sterile water at 25^0C at 290 RPM for 10 min. Leaves were removed and the water was centrifuged at 3220 g for 20 min. All but 5 mL of undisturbed water was removed and the remainder was centrifuged again. Last 1 mL water was transferred to microfuge tube and centrifuged at 14550 g for 5 min at room temperature. The pellet obtained was used for RNA extraction using Ambion RiboPure Bacteria kit (Life technologies, Carlsbad, CA) according to manufacturer's instructions. cDNA construction was done by two-step reaction using TaKaRa RNA PCR kit (Clontech, Montain View, CA) and PCR products were observed on agarose gel. Specifically, expression of coronatine biosynthetic genes, cmaA and cmaB, was checked. 16S rRNA was used as the control for constitutive expression.

2.3.10 Detection of Coronatine Production by Pseudomonas syringae In Vitro

Pst DC3000, *Pst* DC3118, *Pst* DB29, *Pst* DC3000 *hrpA*$^-$, *Pst* DC3000 *hrcC*$^-$ and *Pst* DC3000 *psyI*$^-$/*R*$^-$ were grown in low salt LB medium overnight with appropriate antibiotics and 1 x 10^8 cells from each culture were transferred to liquid HSC medium (nutrients per liter: 1.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 4.1 g KH_2PO_4, 3.6 g $K_2HPO_4.3H_2O$, 0.3 g KNO_3, 10 mL of 2mM $FeCl_3$. Nine parts of this solution was amended with one part of 20% glucose), an optimized coronatine (COR) medium (Palmer and Bender, 1993) at 18^0C for 24 h. COR was extracted from the culture supernatant as previously described (Palmer and Bender 1993) using the abbreviated extraction protocol. Presence of coronatine was analyzed by HPLC on an ASTEC (Whippany, NJ, USA) C8 column (4.6 x 250 mm, 5 µm) at 208 nm. Isocratic separations were performed using a 0.05%

trifluoroacetic acid / acetonitrile (60/40) mobile phase with a flow rate of 1.0 ml.min^{-1}. The injection volume was 5 µl and the column temperature was 25^{0}C. Calibration curves for COR were obtained with commercially available preparation (Sigma Aldrich, Saint Louis, MO). The amount of COR produced was expressed as a function of protein concentration. The cells used for COR extraction were lysed by suspending in 1 M NaOH followed by boiling and freezing three times, and the protein content in bacterial cell lysates was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

2.3.11 Statistical Analysis

Statistical significance of the results was calculated using 2-tailed Student's *t*-test. All experiments reported here were repeated at least two times with similar results.

Table 1 Sequence of primers used to detect transcript of hormone responsive and coronatine biosynthesis genes.

Gene	Primer	Sequence (5'-3')	Size (bp)
ACT8 (At1g49240)	Forward	TTCCGGTTACAGCGTTTGGAGAGA	87
	Reverse	AACGCGGATTAGTGCCTCAGGTAA	
JAZ1 (At1g19180)	Forward	CGTGTAGTCGATTGAGTCAGTATCTAAAAGAGAACG	180
	Reverse	CGGTTTAACATCTTGAACCATGGAATCCATGTTAG	
JAZ2 (At1g74950)	Forward	CTTCTTCCTCTTCCTCTGGGACCAAAG	127
	Reverse	CATCAAACACCATAACTCGACCACCG	
JAZ3 (At3g17860)	Forward	CGGTTCAGTTTGTGTTTACGATGA	97
	Reverse	CGAAAAGACTTGAGGCATAGAGGA	
JAZ4 (At1g48500)	Forward	GAGTTTAGCATCCACGCAACAA	110
	Reverse	TGCGTTTCTCTAAGAACCGAGCCA	
JAZ5 (At1g17380)	Forward	CAGGGCATTCCAAAGGCGAACC	115
	Reverse	CTTTCCCTCCGAAGAATATGGTCAGC	
JAZ6 (At1g72450)	Forward	CTATTGGTGAGGCCTCTACTTCTACCG	110
	Reverse	CCAAAGAATATGGTCAACTGTGAATTTCCAGAC	

Table 1 continued.

JAZ7 (At2g34600)	Forward	GATGCAAACAAAATGCGACTTGGAACTTCG	129
	Reverse	TGGTTAATATCTGAGATTCTTGCTTTGGTTGTG	
JAZ8 (At1g30135)	Forward	CAGCAAAATTGTGACTTGGAACTTCGTC	129
	Reverse	GTTATTCTTTGAGATTCTTCATTTGGTTGTGG	
JAZ9 (At1g70700)	Forward	TCATTCAATGCAGCTCCTCGT	64
	Reverse	TCCGAGCTTGAGGGATGAAG	
JAZ10 (At5g13220)	Forward	CGCTCCTAAGCCTAAGTTCCAGAAATTTCTC	119
	Reverse	GTTTCCAGTGGAAGCTAACAGCGATTTG	
JAZ11 (At3g43440)	Forward	GTTCTGTTTCCGCCGGACTTGAC	120
	Reverse	CCATTGAAGACTCTACAACTCCCACCAAAG	
JAZ12 (At5g20900)	Forward	CTATTGCAAGGAGGCATTCGCTTCAAC	110
	Reverse	GTTGGGACATCTGTCTTTTTGAAGTCTGAAG	
LOX3 (At1g17420)	Forward	GGATGCTTTTGCTGATAAAATTGGTCGAAAC	125
	Reverse	CGATTTCTTTGACCAATCCTTTAAAACTGCTGC	

Table 1 continued.

OPR3 (At2g06050)	Forward	GCGTTGGCAGAGTATTATGCTCAAC	122
	Reverse	CTTGTTCATCTGAATAGATTCCAGGCACATG	
PR-1 (At2g14610)	Forward	CTTGTTCTTCCCTCGAAAGCTCAAGATAGC	116
	Reverse	GAGCATAGGCTGCAACCCTCTC	
PR-2 (AT3G57260)	Forward	CTCACCACCAATGTTGATGATTCTTCTCAG	120
	Reverse	GATGGACTTGGCAAGGTATCGCCTAG	
cmaA	Forward	TTATCAACAGCTCCAGACGCAGG	542
	Reverse	GCAGCGGTACCCAAACTTCAAAC	
cmaB	Forward	AGACCATCCGAGCACAACTGTTC	389
	Reverse	TAGTTCATTTGCTTGTGGCTGCC	
16S rRNA	Forward	AGAGTTTGATCCTGGCTCAG	1500
	Reverse	CGGTTACCTTGTTACGACTT	

2.4 Results

2.4.1 Bacterium-triggered Stomatal Closure is Compromised under High Relative Humidity.

To assess the effect of RH on bacterium-induced stomatal closure, the *P. syringae* strain DC3118 that is not able to re-open stomata was chosen for these experiments. Col-0 plants were surface-inoculated with the COR-defective mutant *Pst* DC3118 and incubated at two different RH conditions. Bacterium treated leaves incubated at 60% RH showed significant decrease in stomatal aperture when compared with the control, water treated leaves. At >95% RH, bacterium-triggered stomatal closure in intact leaves was abolished (Fig. 2.2A). These results indicate that stomatal immunity is not effective under high RH condition. Suppression of stomatal immunity is seen as early as 30 min-1 h after infection. Plants infected with *Pst* DC3118 at 60% RH were virtually symptomless throughout the duration of the experiment (three days), similar to mock-inoculated control plants. However, plants infected under >95% RH showed disease symptoms in their leaves (Fig. 2.2B), similar to the symptoms observed on infection with COR-producing *Pst* DC3000.

Recently, syringolin A produced by *Pss* B728a has been described as virulence factor that facilitates bacterial penetration into its host, common bean (*Phaseolus vulgaris*) (Schellenberg et al. 2010). Hence, the effect of RH on stomatal immunity in this pathosystem was assessed. Similar to what we have observed with Arabidopsis and *Pst* DC3118, bean seedlings (cultivar Beluga) infected with the syringolin-deficient mutant *Pss syl*$^{-}$ under >95% RH failed to close stomata (Fig. 2.3A). RH had little effect on the wild type *Pss* B728a population size in the apoplast of bean leaves with lesser population observed at high RH (Fig. 2.3B). *Pss* B728a is a very efficient epiphyte and it has been shown previously that high RH is conducive for formation of high numbers of bacterial aggregates

on the leaf surface (Monier and Lindow, 2004). This might be the reason for lesser penetration in the apoplast and higher survival as an epiphyte. In contradiction to this, *Pss syl*$^-$ population in the apoplast of the surface-infected leaves was 10,000-fold higher under high RH as compared to that of under low RH (Fig.2.3C). Furthermore, *Pss syl*$^-$ infected bean leaves under high RH showed brown spots characteristic of *Pss* B728a infection as early as 24 h post infection (Fig. 2.3D, E). These results suggest that high RH promotes disease at least in part by interfering with stomatal immunity, which may be a common phenomenon in plant-bacterium interactions.

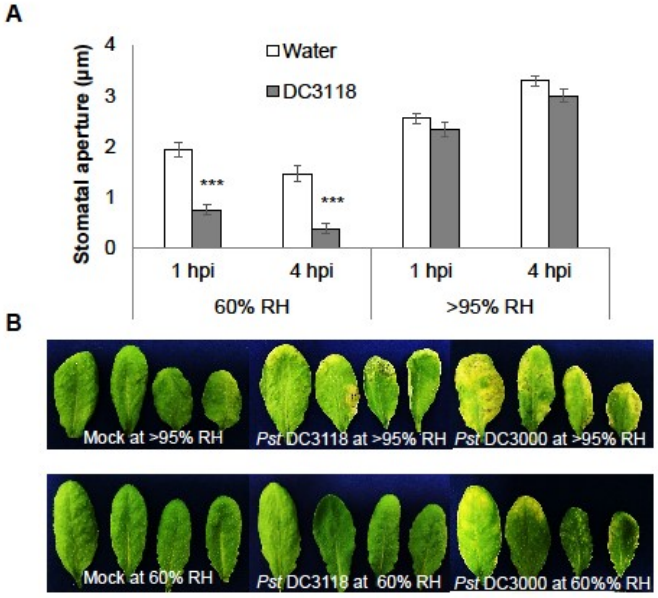

Figure 2.2 Bacterium-triggered stomatal immunity is compromised under high relative humidity (RH) in Arabidopsis. **A,** Arabidopsis stomatal response to *Pst* DC3118 under varying RH. Wild-type Col-0 plants were dipped into bacterial suspensions (1 x 10^8 CFU.ml^{-1}) of *Pst* DC3118 (*cor*$^-$ mutant) or water control (mock inoculation) and stomatal aperture width was measured 1 h and 4 h post inoculation. Results are shown as the mean (n≥60) ± SE. Statistical significance between the means (adjacent bars) was calculated with two-tailed Student's *t*-test (*** = p <0.001). **B,** Disease symptoms observed in Col-0 plants three days after surface-inoculation with *Pst* DC3118 or *Pst* DC3000 (1 x 10^8 CFU.ml^{-1}). Note that some necrosis and yellowing on the leaves appeared only on leaves inoculated with *Pst* DC3118 kept at >95% RH and symptoms become severe with virulent pathogen *Pst* DC3000 at >95% RH.

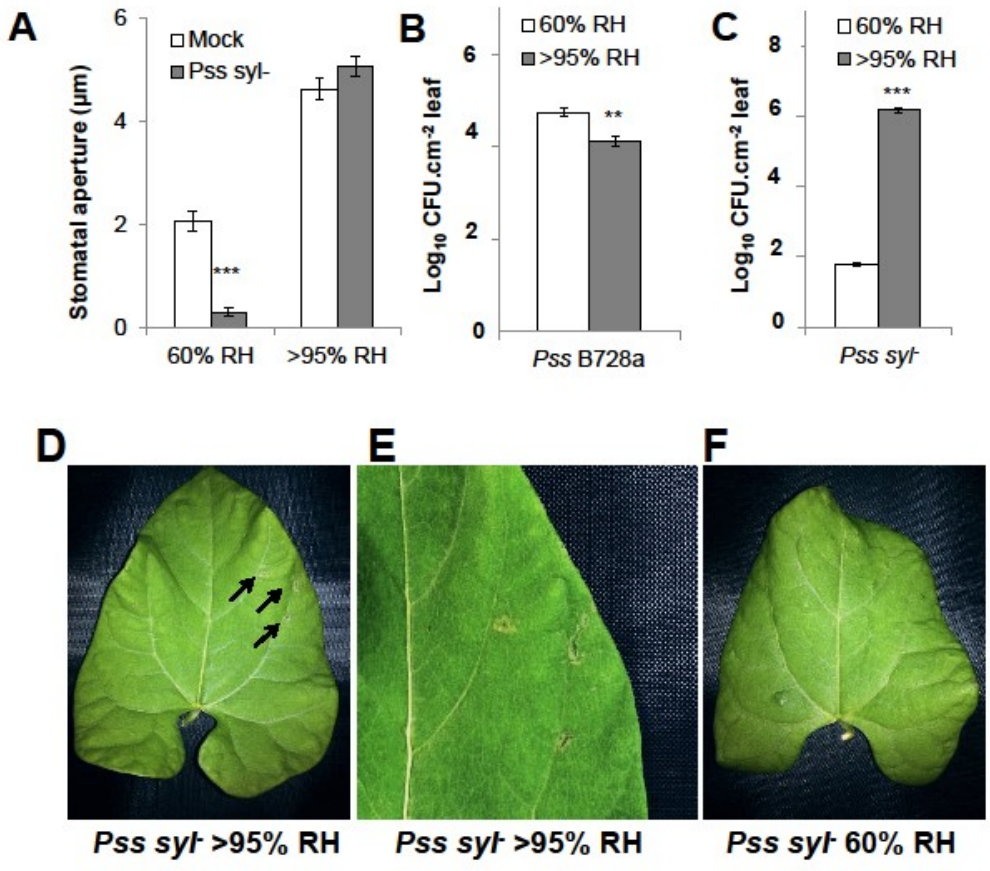

Figure 2.3 Bacterium-triggered stomatal immunity is compromised under high relative humidity (RH) in common bean. **A,** Bean plants were dipped into bacterial suspensions (1 x 10^8 CFU.ml^{-1}) of *Pss syl-* (syringolin A mutant) or water control (mock inoculation) and stomatal aperture width was measured 3 h post inoculation. Results are shown as the mean (n≥60) ± SE. **B** and **C,** Populations of wild type *Pss* B728a (**B**) and syringolin A mutant (**C**) bacteria in the apoplast of bean plants infected under 60% (white bars) and >95% RH (grey bars) at 24 h post inoculation. Statistical significance between the means (adjacent bars in all graphs) was calculated with two-tailed Student's *t*-test (** = p <0.01, and*** = p <0.001). **D, E, F,** Wild-type Beluga seedlings were dipped into bacterial suspensions (1 x 10^8 CFU.ml^{-1}) of *Pss syl-* and symptoms were recorded 24 h after inoculation at two RH levels, >95% (**D** and **E**) and 60% (**F**). Note that necrotic spots appeared only on leaves inoculated with *Pss syl-* kept at >95% RH.

2.4.2 Jasmonic Acid Biosynthesis and Signaling is Up-Regulated by High RH in Whole Leaves.

To determine whether stomatal opening by high humidity correlates with up-regulation of JA signaling pathway, the expression of JA-responsive genes in Col-0 leaves was assessed after exposing plants to high RH (>95%). All genes reported here were confirmed to be expressed in guard cells by direct RNA sequencing (Obulareddy et al. 2013) and microarray analysis (Wang et al. 2011). Because some JA-regulated genes also respond to touch (Chehab et al. 2012), plants were not moved during the experimentation time and high humidity was achieved by spraying the leaves with a fine mist of sterile water and covering plants with humidity domes pre-sprayed with sterile water. It was observed that high RH up-regulates two genes involved in JA biosynthesis, *LOX3* and *OPR3* (Stintzi and Browse, 2000) as early as 15 min after exposure to >95% RH in whole leaves. The expression levels of these two genes returned to basal level at 4 h and were significantly below the basal level at 8 h under high RH (Fig. 2.4). The rapid induction of these genes were dependent on the presence of COI1, however repression of *LOX3* was still detected in *coi1-1* mutant plants (Fig. 2.4), indicating that gene repression might occur by another pathway. To further confirm that JA perception and signaling is required for high RH-induced gene expression, the expression levels of three *JAZ* genes that are known to be induced by JA (Chung et al. 2009) were assessed in whole leaves. *JAZ1*, *JAZ8*, and *JAZ10* were significantly induced by exposing plants to high RH and the induction was compromised, at least partially, in *coi1-1* mutants plants (Fig. 2.4). Similar to *LOX3*, *JAZ1* and *JAZ10* were repressed 8 h after exposure to high RH in both Col-0 and *coi1-1* plants.

There are 12 *JAZ* genes annotated in the Arabidopsis genome that were identified by sequence homology (Chini et al. 2007; Thines et al. 2007). All *JAZ* genes seem to be regulated by relative humidity. Similar to other reported observations, the kinetics of

expression of each *JAZ* gene in whole leaves differed in response to various treatments such as high air humidity (Fig 2.5), *Pst* DC3000 inoculation (Demianski et al. 2012), and JA/wounding (Chung et al. 2008). Nonetheless, all *JAZ* genes, but *JAZ8* and *JAZ9*, were repressed after 8 h of exposure to >95% RH regardless if they were induced or not (Fig 2.5). These results suggest that a possible negative feedback loop may exist (*i.e.* repression of the JA signal in Col-0 in later time points) in addition to other mechanism(s) to repress early JA-induced genes.

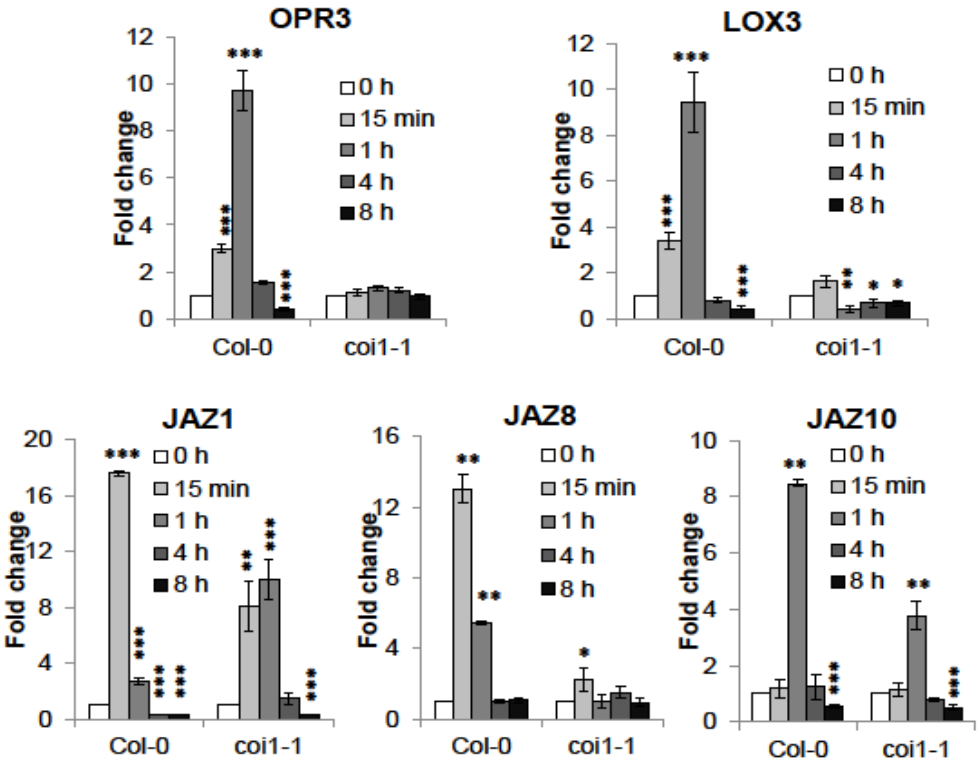

Figure 2.4 JA biosynthesis and signaling is up-regulated in high humidity at early time points in whole leaves. The graphs show fold change expression of the indicated genes after placing plants under >95% RH as compared to plants under 60% RH (0 h). Data points are average (n=3) ± SD. The asterisks above the bars indicate statistical significance in comparison to the 0 h time point as calculated with two-tailed Student's *t*-test (* = p <0.05, ** = p <0.01, *** = p <0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation. Note that error bars for some data points are very small and do not appear in the graph.

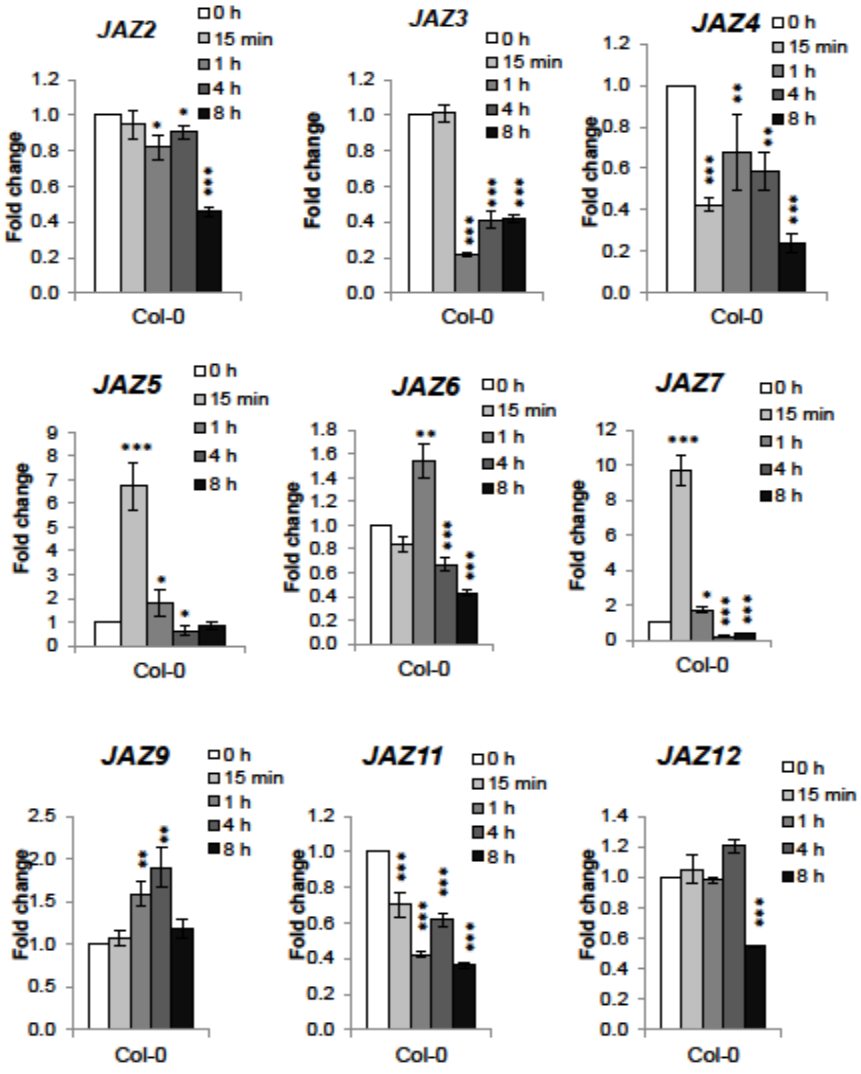

Figure 2.5 Differential regulation of different *JAZ* genes by high RH in whole leaves. Data points indicate the relative expression of indicated genes after placing Col-0 plants under >95% RH as compared to plants kept at 60% RH (0 h). Results are shown as average (n=3) ± SD. The asterisks above the bars indicate statistical significance in comparison to the 0 h time point as calculated with two-tailed Student's *t*-test (* = p <0.05, ** = p <0.01, *** = p <0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation. Note that error bars for some data points are very small and do not appear in the graph.

2.4.3 High Humidity Suppresses the Induction of Salicylic Acid Responsive Genes in Whole Leaves.

JA and salicylic acid (SA) signaling pathways antagonize each other in plants (Van der Does et. al 2013; Kloek et al. 2001) and the SA signaling pathway is required for stomatal immunity as well as apoplastic defenses against *Pst* DC3000 (Kloek et al. 2001; Melotto et al. 2006; Zeng et al. 2010; Zeng et al. 2011). Therefore, the expression of hallmark response genes in the SA signaling pathway (*PR1* and *PR2)* was compared in whole leaves of plants inoculated under a moderate (60%) or high (>95%) RH. Coronatine defective mutant *Pst* DC3118, which can induce SA signaling (Zhao et al.2003; Brooks et al. 2005) for these experiments, was used. At the 60% RH, *Pst* DC3118 significantly induced both *PR1* and *PR2* expression as compared to the mock-inoculated Col-0 and *coi1-1* plants 8 h post-inoculation (Fig. 2.6). Under high RH (>95%) however, *PR1* and *PR2* induction by *Pst* DC3118 was considerably reduced as compared to the expression levels in both Col-0 and *coi1-1* plants inoculated at 60% RH (Fig. 2.7). Repression of these genes continued until 24 h post-inoculation. These results support the notion that high RH suppresses the defense-associated SA signaling in whole leaves, independent of COI1, allowing for increased plant susceptibility to a weak pathogen such as *Pst* DC3118.

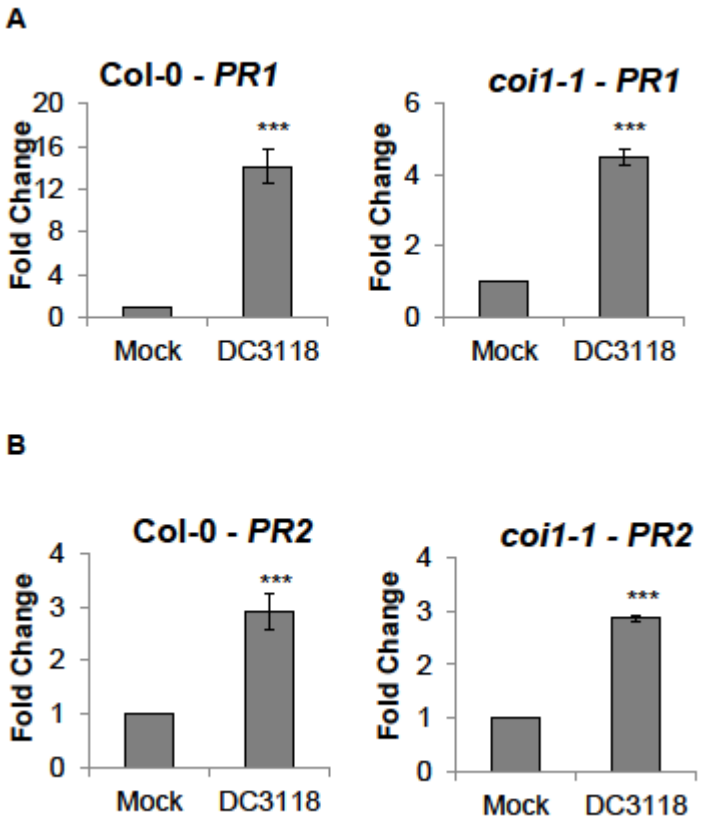

Figure 2.6 *Pst* DC3118 induces of SA-responsive genes in a COI1-independent manner in whole leaves. All graphs show relative expression of the *PR1* (**A**) and *PR2* (**B**) genes in Col-0 and *coi1-1* plants 8 h after dip-inoculation with *Pst* DC3118 under 60% RH. Relative expression was calculated based on the expression levels of mock-inoculated plants, which was considered as 1. Data points are average (n=3) ± SD. The asterisks above the bars indicate statistical significance between the means (mock versus *Pst* DC3118) calculated with the Student's *t*-test (*** = p <0.001).

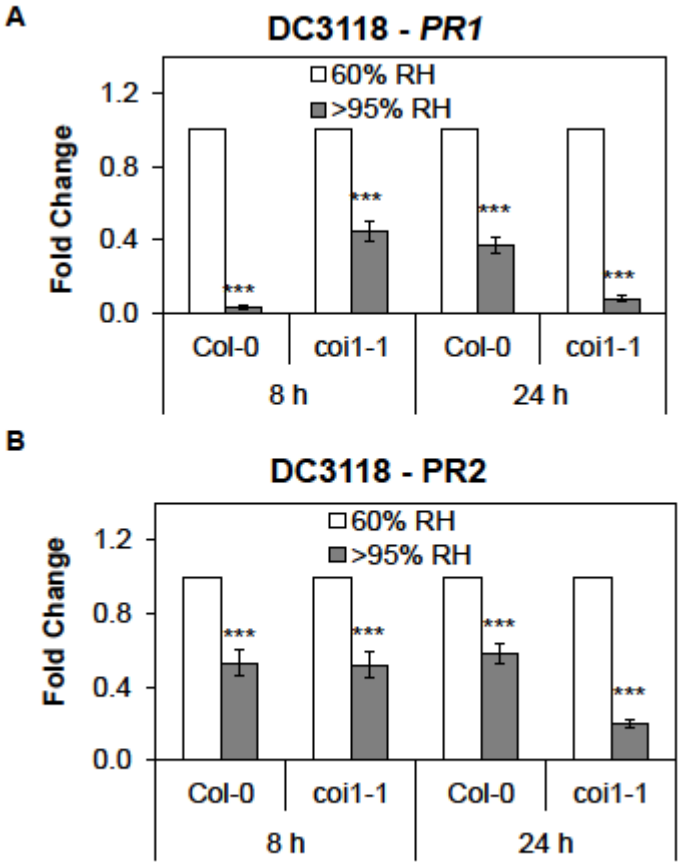

Figure 2.7 High RH represses the induction of SA-responsive genes in whole leaves. The graph shows relative expression of the *PR1* (**A**) and *PR2* (**B**) genes in Col-0 and *coi1-1* plants 8 h and 24 h after dip-inoculation with *Pst* DC3118 under 60% or >95% RH. Relative expression was calculated based on the expression levels of plants kept under 60% RH (white bars), which was considered 1. Data points are average (n=3) ± SD. The asterisks above the bars indicate statistical significance between the means (60% versus >95% RH) calculated with the Student's t-test (*** = p <0.001).

2.4.4 COI1 is Not Required for Suppression of Stomatal Immunity by High RH, but is Important for Complete Opening of Stomatal Aperture.

It was next determined whether the induction of COI1-dependent JA pathway was important for suppression of stomatal immunity by high RH, by assessing stomatal

immunity in *coi1-1* mutant plants. After plants were exposed to treatments, stomatal aperture measurements were obtained directly from intact leaves without further processing of leaf samples to avoid unanticipated responses of mutant plants towards common buffers used to maintain healthy epidermal peels. Similarly to the wild type, *coi1-1* plants close their stomata in response to *Pst* DC3118 at 60% RH, but not at >95% RH (Fig. 2.8A). This means that suppression of stomatal immunity by high RH is seen even in *coi1-1* plants. Surprisingly, *coi1-1* stomata do not fully open when compared to the stomatal apertures of Col-0 plants under either RH levels (Fig. 2.8A). It was further confirmed that *coi1-1* plants have constitutively smaller stomatal aperture by comparing the aperture width in leaves of mutant and wild type plants without any treatment (Fig. 2.8B). Since this difference could be attributed to some defect in guard cell morphology, width of the stomatal complex, guard cell pair size, length of the stomatal complex, size of the stomatal complex, and stomatal density, was checked in both Col-0 and *coi1-1* plants in normal conditions (Fig. 2.8C). It was observed that the width of the stomatal complex is smaller in *coi1-1* as compared to Col-0 (Fig. 2.8D). However, this difference can be attributed only to smaller aperture in *coi1-1*, since the guard cells are of similar size in both genotypes (Fig. 2.8E). The length is also similar, but the size of the entire complex is smaller because of smaller width (Fig. 2.8 F, G). However, the average number of stomata in the leaf is similar in both genotypes (Fig. 2.8H). This suggests that both genotypes harbor similar number and similar type of stoma-forming guard cells, and COI1 is required for keeping wide open stomata. However, COI1 is not required for stomatal closure or high humidity-dependent repression of stomatal immunity.

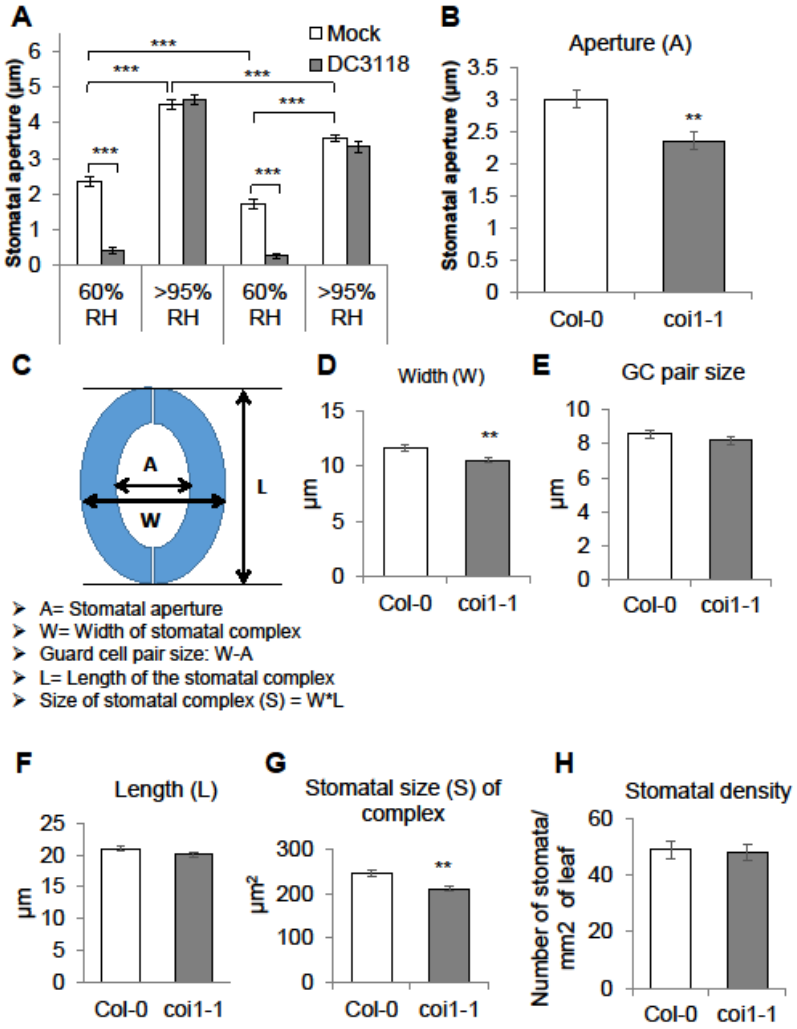

Figure 2.8 Suppression of stomatal immunity by high RH is COI1-independent. **A,** Col-0 and *coi1-1* plants were dipped into bacterial suspensions (1 x 10^8 CFU.ml^{-1}) of *Pst* DC3118 (*cor*⁻ mutant) or control, mock inoculation and stomatal aperture width was measured in intact leaves 4 h post inoculation. Results are shown as the mean (n≥60) ± SE. **B,** Stomatal aperture of Col-0 and *coi1-1* leaves in absence of any treatment. Measurements were performed 3 h after lights turned on to observe naturally open stomata. Results are shown as the mean (n≥60) ± SE. **C,** Diagram of stoma-forming guard cells, showing parameters used for studying guard cell morphology. **D, E, F, G** Morphology parameters measured according to **C.** Results are shown as the mean (n≥60) ± SE. **H,** Average number of stomata/mm^2 of leaf surface counted in Col-0 and *coi1-1*. The asterisks above the bars indicate statistical significance in comparison to Col-0 as calculated with two-tailed Student's t-test (** = p <0.01, *** = p <0.001).

2.4.5 JA-Signaling, but Not JA-Biosynthesis is Induced by High RH in Guard Cells.

It was surprising to observe that COI1 was not required for suppression of stomatal immunity by high RH. So, could there be a possible JA signaling which does not involve COI1? To test this assumption of a COI1-independent JA signaling pathway, expression of three genes, *JAZ1, JAZ8, and JAZ10* was checked in high RH. These three genes were partially induced in whole leaves of *coi1-1* plants (Fig. 2.4), indicating that they could be regulated in the absence of COI1 as well. Hence, their gene expression in guard cells was checked. Leaves exposed to high RH were harvested and guard cells were extracted for gene expression studies. The time points chosen for checking gene expression was 15 min and 1 h, as early induction of these genes was observed in whole leaves (Fig 2.4). Moreover, stomatal response is a very rapid event and the events preceding it should occur in guard cell very early as well. As shown in Fig 2.9A, *JAZ1, JAZ8,* and *JAZ10* were induced within 1 h of transfer to high RH. As compared to signaling genes, JA biosynthesis gene induction was completely dependent on COI1 in whole leaves (Fig 2.4). As seen in Fig 2.9B, *OPR3* is not induced and *LOX3* is only slightly and transiently induced in response to high RH in guard cells. In fact, these genes are repressed within one hour. This indicates that only JA signaling events, and not JA biosynthesis, are up-regulated by high RH in guard cell, leading to opening of stomata.

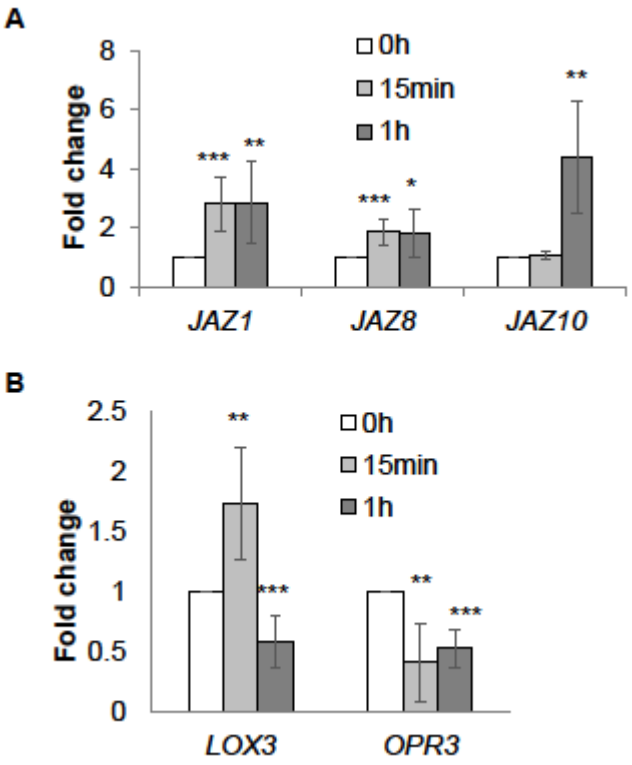

Figure 2.9 JA signaling, but not JA biosynthesis, is up-regulated in high humidity in guard cells. The graphs show fold change expression of the indicated genes after placing plants under >95% RH as compared to plants under 60% RH. Data points are average (n=3) ± SD. The asterisks above the bars indicate statistical significance in comparison to the 0 h time point as calculated with two-tailed Student's *t*-test (* = p <0.05, ** = p <0.01, *** = p <0.001). Significant means above 1 are considered up-regulation and significant means below 1 are considered down-regulation. Note that error bars for some data points are very small and do not appear in the graph.

2.4.6 SA Responsive Genes are Repressed by High RH in Guard Cells.

Leaves exposed to high RH were harvested at 15 min and 1 h after transfer to high RH and guard cells were extracted for studying SA responsive gene expression. This experiment was done without any bacterial treatment to study the effect of only RH on *PR1* gene expression. It was observed that *PR1* is repressed by high RH within 15 min (Fig

2.10A), indicating that SA responsive genes may be regulated even in the absence of biotic stress. To compare with previous results in whole leaves where repression by high RH was observed in the presence of bacteria *Pst* DC3118 (Fig. 2.7), gene expression of *PR1* and *PR2* was checked in guard cells from plants infected with *Pst* DC3118 and then kept in two different RH. In guard cells, the SA-responsive genes *PR1* and *PR2* were repressed by high RH (Fig 2.10B) at 1 h post-inoculation even in the presence of biotic stress, similar to whole leaves (Fig. 2.7). This suggests that SA-defense signaling is down-regulated by high RH in guard cells.

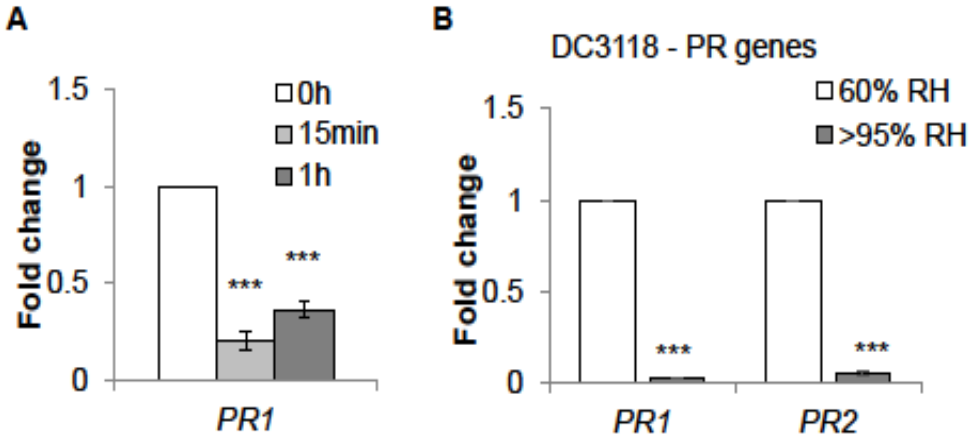

Figure 2.10 High RH represses the induction of SA-responsive genes in guard cells. **A,** The graph shows fold change expression of *PR1* after placing plants under >95% RH as compared to plants under 60% RH (white bar), which was considered 1. **B,** The graph show relative expression of the *PR1*and *PR2* genes in Col-0 1 h after dip-inoculation with *Pst* DC3118 under 60% or >95% RH. Relative expression was calculated based on the expression levels of plants kept under 60% RH (white bars), which was considered 1. Data points are average (n=3) ± SD. The asterisks above the bars indicate statistical significance between the means (60% versus >95% RH) calculated with the Student's *t*-test (*** = p <0.001).

As mentioned previously, infection with *Pst* DC3118 leads to induction of *PR1* and *PR2* genes and up-regulation of SA-signaling pathway in whole leaves at 8 h post infection (Fig. 2.6) in moderate RH. This response was checked in guard cells. For guard cell gene expression, again the time point chosen was 1 h. It was observed that instead of being induced in the presence of *Pst* DC3118, these genes were repressed in the presence of the bacterium (Fig. 2.11A,B). This was surprising since it is reported that *PR* genes are induced in the presence of COR mutant at 24 or 48 h (Zhao et al.2003; Brooks et al. 2005). Why are these genes repressed in guard cells? Since we could clearly observe induction at 8 h in whole leaves (Fig. 2.6), it was checked if regulation of gene expression was dependent on the time of infection by observing *PR1* and *PR2* gene expression in whole leaves at 1 h. As shown in Fig. 2.11C,D, similar pattern of repression of genes in the presence of bacterium is observed. This indicates that at early time points of infection, SA defense may not be active.

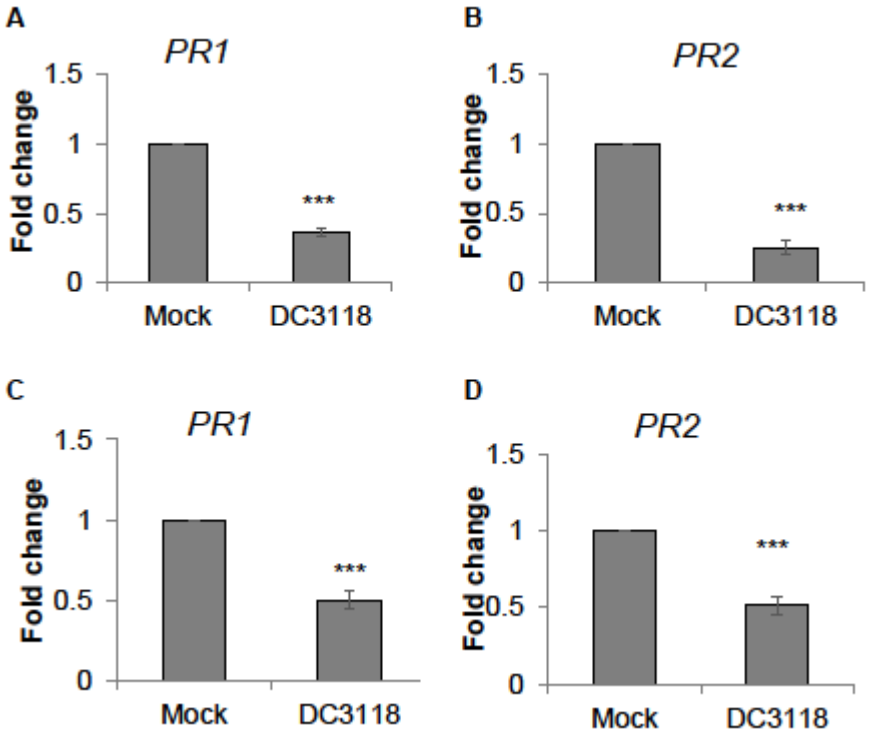

Figure 2.11 SA-responsive genes are not induced at early time point of 1 h in guard cells and whole leaves. The graphs show relative expression of the *PR1* (**A, C**) and *PR2* (**B, D**) genes in Col-0 guard cells (**A,B**) and whole leaves (**C,D**) 1 h after dip-inoculation with *Pst* DC3118 under 60% RH. Relative expression was calculated based on the expression levels of plants treated with water (mock) at 60%RH, which was considered 1. Data points are average (n=3) ± SD. The asterisks above the bars indicate statistical significance between the means (mock versus DC3118) calculated with the Student's *t*-test (*** = p <0.001).

2.4.7 COR Biosynthesis Genes are Activated on the Leaf Surface Independent of Light.

Previous studies showed that *Pst* DC3000 re-opens stomata in a COR-dependent manner within 3 h to 4 h of contact with plant tissue and that COR opens dark-closed stomata. These results raised an immediate question about the timing and location (*i.e.* epiphytic or endophytic) of COR production by the bacterium. Expression of COR biosynthesis genes *cmaA* and *cmaB* was assessed in epiphytic *Pst* DC3000. This was

done by spraying the leaf surface with 1 x 10^8 CFU.ml^{-1} *Pst* DC3000, dislodging and collecting bacterial cells from the leaf after 6 h, and checking gene expression. As shown in Fig.2.12, there was no change in gene expression observed after 6 h in contact with leaf. A different method using reporter gene was used to observe if COR biosynthesis is induced on the leaf surface. The bacterial strain *Pst* DC3000 (pHW01) that contains a reporter plasmid expressing GFP driven by the promoter of the COR biosynthesis gene *cma* (Weingart et al. 2004) was used to monitor the appearance of green florescence, indicative of high-level activation of the *cma* promoter. Intact leaves were used to discard the possibility that the promoter was induced by the content of ripped mesophyll cells. Leaves were floated on bacterial suspension (Fig 2.1) and the appearance of green fluorescent bacterial cells was monitored over a 24 h period. Strong green fluorescence was observed in bacteria (2.42 ± 0.36 bacteria per 0.075mm^2 of leaf) at approximately 4 h after contact with the surface of intact leaf (Fig 2.13B) and at 24 h, the entire leaf surface area was covered with fluorescing cells (Fig 2.13C).

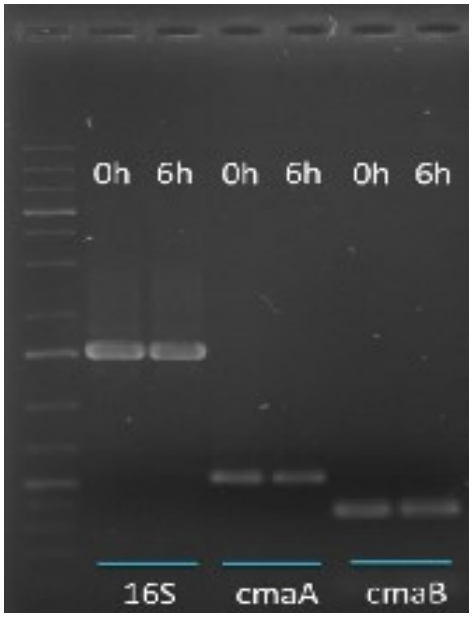

Figure 2.12 Expression of COR biosynthesis genes *cmaA* and *cmaB* in epiphytic *Pst* DC3000. Cells were dislodged from the leaf immediately (0 h) or 6 h after spraying with 10^8 CFU.ml^{-1} of bacteria on leaves and gene expression was assessed.

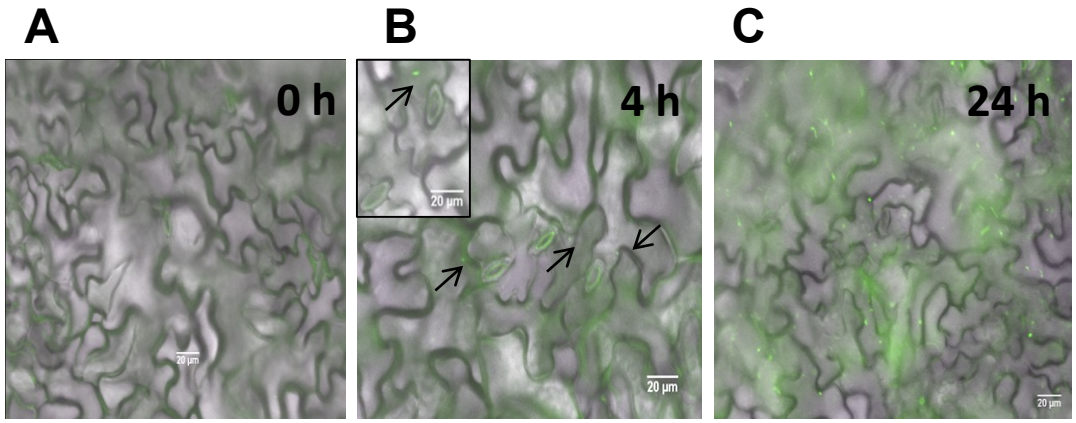

Figure 2.13 Coronatine biosynthesis gene promoter of *Pst* DC3000 (pHW01) is induced in contact with Arabidopsis leaf. Fluorescent microscope images of Arabidopsis leaf surface at 0 h (**A**), 4 h (**B**) and 24 h (**C**) of incubation with *Pst* DC3000 (pWH01). Black arrows show few fluorescing cells at 4 h. Several cells were fluorescing at 24 h.

At night or in dark conditions, stomata of most land plants close. Production and action of COR in such conditions can provide an advantage to the pathogen for infection at night. Other studies in the lab suggested that COR is able to re-open dark-closed stomata (Chitrakar, 2010). To assess whether light is required for the induction of the *cma* promoter in epiphytic *Pst* DC3000, leaves floating on bacterial suspension were kept under constant light or constant darkness and bacterial fluorescence was examined 24 h after exposure to leaves. It was observed that the activation of the *cma* operon was independent of the light regime (Fig 2.14). Taken together, these results provide strong evidence that COR production can occur prior to bacterial penetration through the stomata, independent of the light condition.

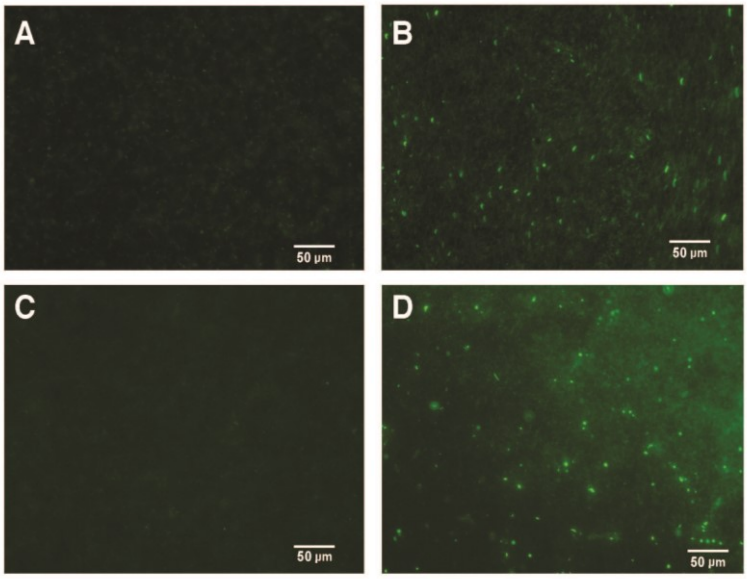

Figure 2.14 Coronatine biosynthesis gene promoter is induced on the leaf surface in a light-independent manner. Fluorescent microscope image of *Pst* DC3000 (pWH01) after incubating the cells with intact Arabidopsis leaf surface under light (**A** and **B**) or darkness (**C** and **D**) for 0 h (**A** and **C**) and 24 h (**B** and **D**).

To further check if light is important for the amount of COR produced by the bacteria, HPLC-based method was used. In order to optimize this method, COR production was first confirmed in strains previously reported to produce COR (Peñaloza-Vázquez et al. 2000), including *Pst* DC3000 and type-three secretion system mutants *Pst* DC3000 *hrpA*$^{-}$, *Pst* DC3000 *hrcC*$^{-}$, and COR mutants *Pst* DB29 and *Pst* DC3118. Total amount of COR was normalized to total protein of the cell culture. The result in Fig. 2.15 is what had been observed previously, with no COR observed with COR mutants, DB29 and DC3118, and COR production observed in varying quantities in wild type *Pst* DC3000 and type-three secretion system mutants, *hrpA*$^{-}$ and *hrcC*$^{-}$.

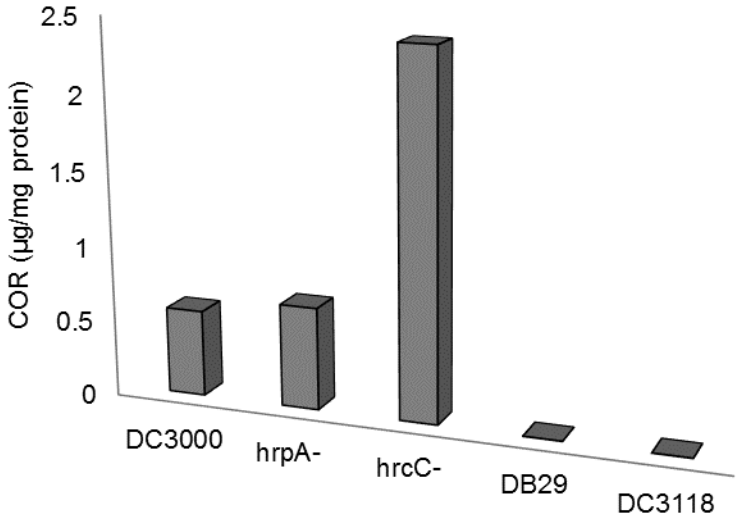

Figure 2.15 Amount of COR produced in vitro in different strains of *Pst* DC3000. Bacteria were grown in COR inducing medium (HSC medium) for 24 h and COR production was estimated by HPLC. COR concentration in bacteria calculated as µg COR per mg of total protein is shown. Data points are shown as mean (n=6).

Using the same protocol, COR production by *Pst* DC3000 was checked when the bacterium was grown in light and dark conditions. It was observed that light regimes have no effect on COR production (Fig. 2.16D). Hence, it can be concluded that light or dark conditions have no effect on induction of coronatine biosynthesis as well as coronatine production.

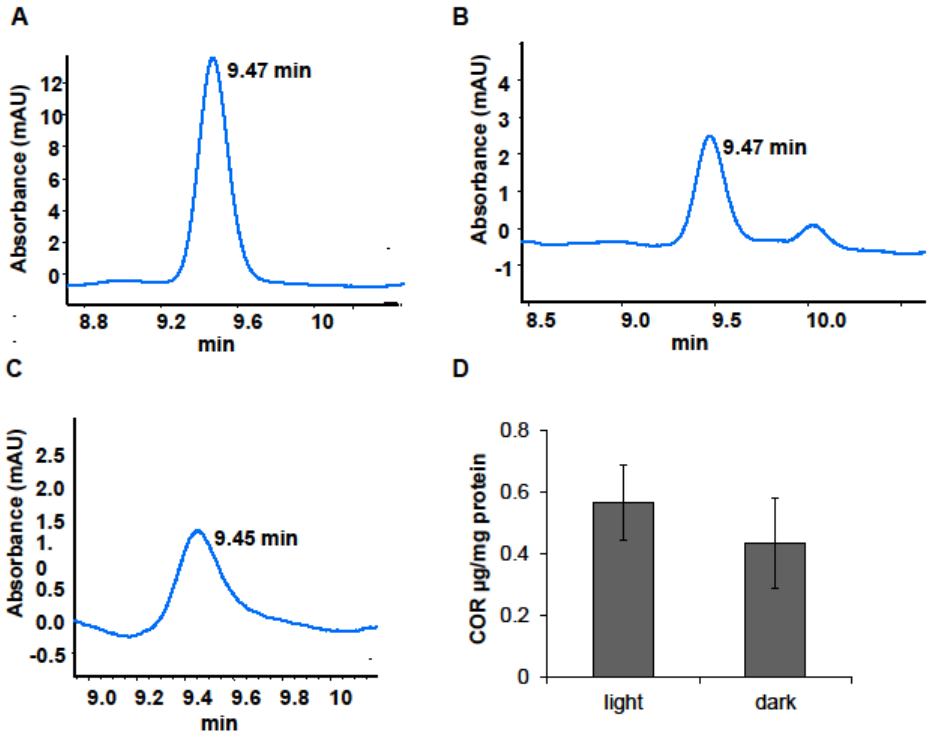

Figure 2.16 *Pst* DC3000 produces comparable amounts of coronatine under light or darkness. *Pst* DC3000 was grown in COR inducing medium (HSC medium) for 24 h in constant light (70-80 µmol.m^{-2}.s^{-1}) or constant darkness and COR production was assessed by HPLC. **A, B,** and **C,** Chromatograms obtained by HPLC showing peaks of COR at the retention time of 9.4 min when the sample injected was 15 µg.ml^{-1} COR (control) (**A**), *Pst* DC3000 grown in light (**B**) or in dark (**C**). mAU; milliabsorbance units at 208 nm. **D,** COR concentration in *Pst* DC300 cells grown in light or dark calculated as µg COR per mg of total protein. Data points are shown as mean (n=6) ± SE.

2.4.8 Lack of AHL-Like Signal does not Decrease COR Action and Production in Pst DC3000.

A study by Lenore Price in our lab indicated that the PsyI/PsyR is a two component quorum sensing system in *Pst* DC3000 with PsyI being responsible for the synthesis of acyl homoserine lactone (AHL) and PsyR being the putative receptor for AHL. The signal is a homolog of acyl homoserine lactone (AHL). The parent strain of the double mutant *psyI⁻/psyR⁻* , *Pst* DC3000, that produces COR, has been observed to form clusters on Arabidopsis leaf surface (Melotto et al. 2006). COR is activated on the leaf surface (Fig. 2.13) and AHL production may be possibly required by the bacteria for cell-to-cell communication to aggregate and penetrate through stomata. Hence, requirement of the AHL homolog for this epiphytic lifestyle for virulence was checked by measuring stomatal immunity and in vitro COR production using *psyI⁻/psyR⁻* mutant. As shown in Fig. 2.17A, stomatal response of Col-0 leaves to *psyI⁻/psyR⁻* mutant was very similar to *Pst* DC3000 with closure of stomata at around 2 h and re-opening at 4 h, possibly indicating the production of coronatine for subversion of stomatal immunity. To investigate this possibility, in vitro production of coronatine was checked which indicated that *psyI⁻/psyR⁻* produces as much or even more coronatine than the wild-type bacterium (Fig.2.17B). This suggests that lack of AHL homolog production did not influence one of the virulence mechanisms of the bacterium, which is production of phytotoxin coronatine.

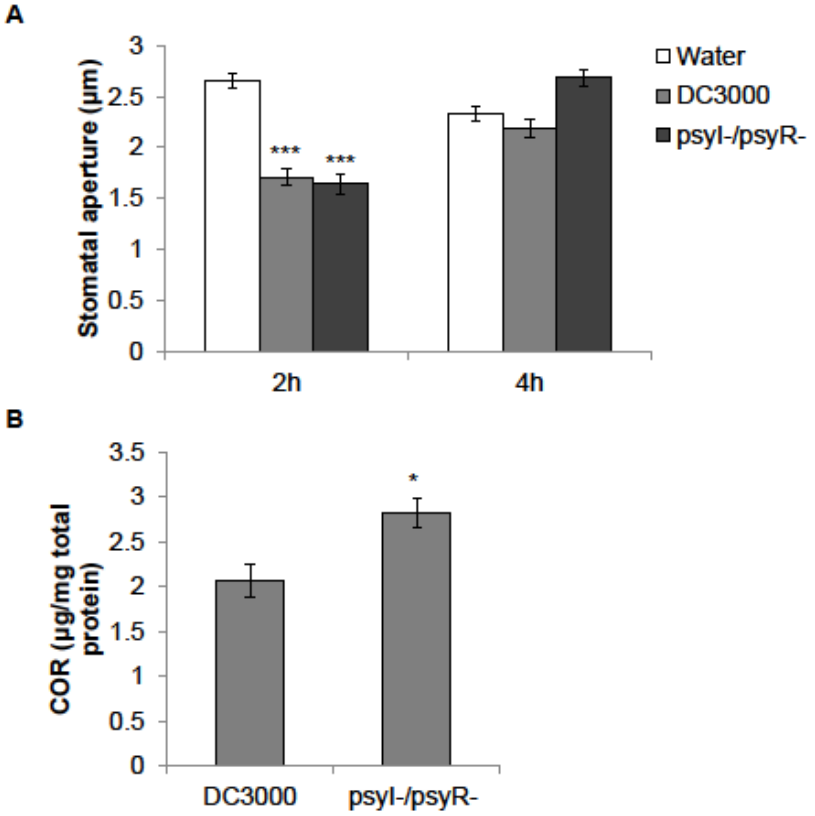

Figure 2.17 *psyI⁻/R⁻* mutant is not deficient in the production of coronatine and subsequent subversion of stomatal immunity. **A**, Wild-type Col-0 plants were dipped into bacterial suspensions (1 x 10^8 CFU.ml^{-1}) of *Pst* DC3000, *psyI⁻/R⁻* mutant or water control (mock inoculation) and stomatal aperture width was measured 2 h and 4 h post inoculation. Results are shown as the mean (n≥60) ± SE. Statistical significance between mock and bacterial treatment was calculated with two-tailed Student's *t*-test (*** = p <0.001). **B**, COR concentration in *Pst* DC300 and *psyI⁻/R⁻* bacteria as µg COR per mg of total protein. Data points are shown as mean (n=6) ± SE. Statistical significance between the means was calculated with two-tailed Student's *t*-test (* = p <0.05).

2.5 Discussion

Environmental factors greatly influence the outcome of plant-pathogen interactions and can favor either partner in the interaction when they come into contact. In this study, the physiological and molecular components involved in stomatal reponse in high humidity were examined. Specifically, the role of jasmonate and salicylic acid signaling in stomatal response to high RH was studied.

It was found that bacterium-triggered stomatal closure is completely abolished under >95% RH favoring bacterial penetration of otherwise low-virulence bacteria, the COR-deficient *Pst* DC3118 and the syringolin A-deficient *Pss* B728a. It has been known for years that severe outbreaks of bacterial disease in crop plants are often associated with periods of heavy rainfall or high humidity. Mechanical wounding of plant tissues due to rain might be one way that allows pathogens to bypass the stomatal route and gain access to the plant interior. Formation of large bacterial aggregates under high humidity on the leaf surface (Monier and Lindow, 2004) and splashing of bacteria during rain may also contribute to the spreading of disease at a higher rate. Another possibility, however, is that stomatal defense is highly compromised during periods of high rainfall. This would allow unrestricted entry of pathogens into the plant and consequently produce more severe disease. All these results provide direct evidence that high humidity favors internalization of bacteria (*e.g.*, *Pst* DC3118 and *Pss syl*$^{-}$) that otherwise cannot actively subvert stomatal immunity.

The fully virulent COR-producing *Pst* DC3000 activates JA signaling pathway in a COR-dependent manner (Zhao et al. 2003, Brooks et al. 2005), which contributes to suppression of stomatal and apoplastic defenses and promotion of disease development (Melotto et al. 2006; Kloek et al. 2001). Therefore, it was reasoned that high RH might induce JA signaling and promote plant susceptibility to COR-deficient bacteria. Indeed, a

major observation in this study was that JA primary response genes (Chung et al. 2008) are up-regulated as early as 15 min after exposing plants to high RH (>95%). Induction of the jasmonic acid pathway by high RH correlated well with decreased stomatal closure and the high degree of susceptibility in plants inoculated with the low-virulence pathogen *Pst* DC3118, suggesting the high RH might compromise plant defenses at least in part by the activation of the JA signaling pathway. Comparison of gene expression in whole leaves and guard cells revealed that JA signaling activation in guard cell was independent of COI1. This was supported by observing stomatal response in *coi1-1* plants, where high humidity suppressed stomatal immunity even in the absence of COI1. COI1-independent and JA-dependent signaling pathway has been previously proposed and induction of some *JAZ* genes in *coi1* plants has been reported (Stotz et al. 2011). In addition to this, *P. syringae* infection in *coi1-1* plants also leads to induction of JA-regulated genes, indicating that JA response gene can be activated downstream or independent of COI1 (Chen et al. 2002). However, COI1 is important for larger stomatal apertures, which suggests a role in natural stomatal opening. This is also consistent with previous studies showing that COR, the functional and structural mimic of JA-Ile, and which requires COI1 for its function of up-regulation of JA, is responsible for opening of stomata (Melotto et al. 2006; Zhang et al. 2008). Comparison of JA-responsive gene expression in whole leaves and guard cells also indicates that JA biosynthesis is not induced, but is repressed in guard cells. Induction of JA biosynthesis genes occurs as a feedback loop to replenish JA-Ile binding to COI1. However, COI1-independent mechanism is observed in guard cell, suggesting that replenishment of JA biosynthesis proteins may not be required. However, the repression observed can indicate the requirement to fine tune the response. These results also suggest that JA-responsive genes are regulated in whole leaves and guard cells differently by high RH. Although high RH seems to regulate these in the entire leaf tissue, it seems

that guard cell possesses a different pathway which is targeted by high RH. Guard cell-autonomous abscisic acid (ABA) synthesis pathway has been described recently (Bauer et al. 2013a). This indicates that signaling in guard cells can also occur independent of other cell types.

It has been shown that JA signaling antagonizes SA signaling in the Arabidopsis-*P. syringae* pathosystem (Kloek et al. 2001). SA-dependent phenotypes are suppressed in plants grown under high RH (Yoshioka et al. 2001) and SA-dependent activation of *PR* genes is suppressed 24 h after shifting plants to high RH (Zhou et al. 2004). In this study, it was observed that *Pst* DC3118 activation of *PR* genes is suppressed in whole leaves by high RH as early as 8 h post treatment and can last at least 24 h. These findings were extended and it was demonstrated that suppression of SA-dependent responses does not require COI1. Collectively, these results support the idea that Arabidopsis immunity, including stomatal defense, against *Pst* DC3000 is decreased under high humidity conditions by early activation of JA signaling and inhibition of SA signaling. These two signaling pathways seem to be independent or the cross-talk is downstream of COI1 and/or at later time points. This might be another example in which the contribution of each hormone and timing of signaling determines the outcome of the plant-pathogen interaction (Gimenez-Ibanez and Solano, 2013). Suppression of SA signaling was also observed in guard cells. Since, stomatal response to high humidity is very rapid with respect to both the phenotype of stomatal aperture (30 min to 1 h) as well as JA signaling activation (15 min to 1h), *PR1* gene expression was checked at 1 h and indeed, suppression of the gene was observed. To observe *PR1* gene suppression, it is a common practice to induce it first with biotic stress. Interestingly, it was seen that this gene was repressed even in the absence of biotic stress in guard cells, suggesting direct regulation of this gene by high RH. On addition of biotic stress with *Pst* DC3118 as well, both *PR1* and *PR2 genes* were

repressed in high RH as early as 1 h. Surprisingly, it was observed that *PR1* and *PR2* are not induced by bacterial treatment in 60% RH suggesting that this defense response must be acting only at later time stage in the infection. Previous studies have demonstrated induction of these genes in response to bacterium, but gene expression was assessed only at later time points (Zhao et al.2003; Brooks et al. 2005). Although JA and SA pathways are antagonistic (Van der Does et. al 2013, Kloek et al. 2001), it is possible that there is no cross-talk between JA and SA signaling in guard cells, since simultaneous induction of JA and repression of SA were observed. Alternatively, if there is a cross-talk, it should be downstream or independent of COI1 and extremely fine-tuned with respect to time. Hence, a model can be proposed where high RH up-regulates a COI1-independent JA signaling pathway and represses SA signaling in guard cells, leading to stomatal opening (Fig. 2.18).

Apart from these hormones, high RH has been shown to regulate abscisic acid (ABA) pathway. There have been extensive studies to show involvement of ABA in drought and stomatal closure (Montillet and Hirt, 2013). Low RH has also been shown to induce ABA biosynthesis, leading to stomatal closure (Bauer et al. 2013a). In contrast, high RH induces genes for catabolizing ABA, thus inhibiting stomatal closure (Okamoto et al. 2009). Identification of core genes common to low RH and ABA treatment by transcriptomic studies indicate that ABA signaling and low RH may utilize the same pathway for stomatal closure (Bauer et al. 2013b). This indicates that environmental factors can regulate several signaling pathways. Whether this occurs because of hormonal cross-talk is not known yet. Other than abiotic factors, pathogens secrete a variety of molecules like phytotoxins and effectors that target plant cell components and have been implicated in stomatal re-opening (Fig.2.18). Pathogen secreted molecules like COR from *Pst* DC3000 (Melotto et al. 2006), DSF from *Xanthomonas campestris* (Gudesblat et al. 2009), syringolin from *Pss* B728a (Schellenberg et al. 2010), HopX1 from *Pst* DC3000 (Gimenez-Ibanez et al. 2014), and

fusicoccin from *Fusicoccum amygdali* (Turner and Graniti, 1969) modulate guard cell signaling at different points to cause stomatal opening. Some plant cell targets that are regulated to cause stomatal opening are NAC transcription factors (Montillet et al. 2013), lectin receptor kinase LecRK-V.5 (Desclos-Theveniau et al. 2013), and RIN4 (Liu et al. 2009). It remains to be studied if high RH is able to modulate these plant cell targets.

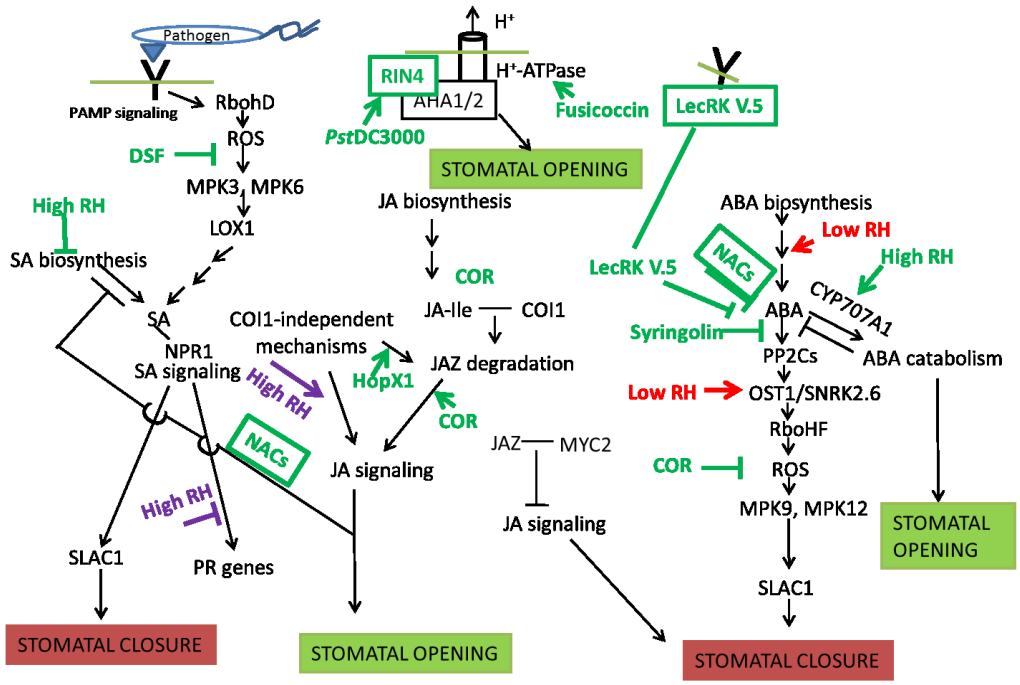

Figure 2.18 Guard cell signaling events involved in regulation of guard cell movement resulting in either open or closed stomata. PAMP recognition leads to signaling involving ROS production production through the NADPH oxidase isoform RbohD, MAP kinase (MPK3 and MPK6) signaling, resulting in activation of LOX1, and leading to SA accumulation and stomatal closure mediated by SLAC1 anion channels. DSF from *Xanthomonas* and high RH can interfere in this. *Pst* DC3000 is able to re-open stomata by activation of RIN4, by secreting HopX1 effector affecting the JA pathway, and by secreting COR interfering with JA, ABA, and SA (through NAC activation) pathway. ABA signaling is modulated by both low and high RH as well as syringolin from *P. syringae* pv. *syringae.* NAC proteins, LecRK V.5 receptor kinase, and RIN4 are plant cell components that positively regulate stomatal opening. Colored components indicate factors involved in stomatal regulation, with green text favoring stomatal opening and red text inducing stomatal closure. Purple text represents regulation of stomatal immunity suggested by my study. Texts in green boxes are plant cell components involved in stomatal opening.

Crude extracts and apoplastic fluids of tomato leaves have been shown to induce COR biosynthetic genes in *P. syringae* (Li et al. 1998). Additionally, the COR biosynthesis operon, *cfl/cfa* is induced 6 h after bacterial infiltration into Arabidopsis leaves (Boch et al. 2002). COR is necessary to overcome stomatal immunity and this activity was observed within 4 h of bacterial contact with intact leaves (Melotto et al. 2006). However, the expression of COR-biosynthetic genes in epiphytic bacterial populations has not been reported. In this study, a reporter gene system (*cma-gfp* in pHW01) was used to estimate the timing of COR production. The results show that a high level of *cma-gfp* expression, as indicated by GFP fluorescence, is detectable within 4 h of bacterial contact with the leaf surface. To the best of my knowledge, this is the first time that COR biosynthetic gene expression has been shown to be induced in the epiphytic phase of the pathogen. COR activation and production is independent of light conditions or presence of AHL-like signal. Although the involvement of COR in epiphytic fitness is not investigated in depth here, the possibility of COR helping epiphytic survival of *Pst* DC3000, in addition to mediating stomatal opening, cannot be ruled out. Indeed, the involvement of the exopolysaccharide alginate and the phytotoxin mangotoxin in epiphytic fitness of *P. syringae* has been described (Arrebola et al. 2009; Yu et al.1999). In the future, it would be important to identify the environmental signal(s) that control COR production on the leaf surface and to investigate whether COR mediates additional aspects of bacterial interactions with the plant surface.

2.6 Conclusion

Disease-favoring environmental condition, high relative humidity, abolishes stomatal immunity in Arabidopsis and bean plants. High humidity acts in part by up-regulating jasmonic acid signaling and suppressing salicylic acid signaling marker gene expression. The targets suggested by this study have been indicated by purple arrows in Fig 2.18. In addition, coronatine biosynthesis is induced on the leaf surface and coronatine production is not affected by light conditions, thus providing an advantage for the pathogen to infect at night.

Chapter 3

Arabidopsis Defense Responses against Human Pathogen Infection

3.1 Abstract

Recently, there have been studies demonstrating active survival mechanisms by certain human pathogens inside and on plants, leading to food poisoning-related outbreaks. A large number of such outbreaks have been associated with two major enteric human pathogens, enterohemorrhagic *Escherichia coli* and *Salmonella enterica*. In this study, it is demonstrated how stomatal and apoplastic immunity in the model plant, *Arabidopsis thaliana* is modulated in the presence of *Salmonella enterica* serovar Typhimurium SL1344. It was observed that stomatal immunity is compromised in the presence of SL1344. In addition to this, other components of defense system like callose deposition and defense gene expression are also compromised. High-throughput transcriptomic studies revealed plant genes that are modulated in the presence of SL1344 in the apoplast. This suggests a possibility that not only plant pathogens, but some human pathogens are also able to disrupt parts of the plant immune system. These studies can be explored further to aid prevention of food-borne outbreaks right at the first step of colonization.

3.2 Introduction

A recent study by Scallan et al. (2011) reported that non-typhoidal *Salmonella* spp. are the leading cause of hospitalizations and deaths in the USA every year due to food contamination. Apart from the direct effect on human health, economic losses incurred by recalling contaminated food products are huge. The eight-day recall of spinach in 2006 cost $350 million to the US economy (Hussain and Dawson 2013). It should be realized

that this is not the loss of one individual; several growers, workers, distributors were at loss. This is a common scenario for any multistate foodborne outbreak. Additionally, the skepticism of the general public towards consumption of a particular food product can lead to deficiency of an important food source from the diet. Economic analysis shows that money spent on prevention of foodborne outbreak by producers is much less than the cost incurred after the outbreak (Ribera et al. 2012). Contamination of plants can occur at any step of food production while the food gets from farm to the table. Both pre-harvest and post-harvest steps are prone to contamination. Contaminated irrigation water, farm workers with limited means of proper sanitation, fecal contamination in the farm by animals can expose the plants to human pathogens before harvest of the edible parts. After harvest, unclean modes of transportation, contamination while processing and bagging can occur (Lynch et al. 2009). Mechanical damage to food during transport can dramatically increase the population of human pathogens surviving on the surface as studied by Aruscavage et al. (2008). Several solutions for preventing contamination have been proposed and used. For example, cleaning and sanitization of leafy greens is usually done before packaging to lower surface pathogen numbers (Lynch et al. 2009). However, some studies are indicating protection of these human pathogens inside plant tissue from sanitization (Seo and Frank, 1999; Saldana et al. 2011). Thus, understanding the biology of this interaction is now crucial to prevent survival or colonization of human pathogens in plants.

As mentioned previously, components of the plant defense system are important to ward off invaders, both plant and human pathogens alike. Human pathogens, like plant pathogens, are also recognized by the plant immune system via conserved microbial signatures. For example, flagellin has been shown to be an important PAMP of *S. enterica* (Garcia et al. 2013; Meng et al. 2013) and *E. coli* O157:H7 (Seo and Mathews, 2012), recognized by the plant receptor FLS2. Purified LPS from *S. enterica* and *E. coli* induces

strong stomatal closure in Arabidopsis (Melotto et al. 2006). However, some successful plant pathogens are able to overcome such defenses using several virulence factors. For example, stomatal immunity prevents bacterial internalization; however, coronatine and syringolin A produced by *Pst* DC3000 and *Pss* B728a, respectively, re-opens closed stomata (Melotto et al. 2006; Schellenberg et al. 2010). But human pathogens like *E. coli* O157:H7 (hereafter referred to as O157:H7) are unable to do so, resulting constitutive activation of stomatal immune response (Melotto et al. 2006; Thilmony et al. 2006; Fig. 3.1). This leads to an understanding that human pathogens are able to activate plant defenses through recognition of conserved pathogen associated molecular patterns (PAMPs), and may be largely restricted to the plant surface. This has a direct implication for food safety measures. However, recent studies have been able to detect bacteria inside the plant, after surface inoculation with *S. enterica* (Gu et al. 2011; Golberg et al. 2011; Barak et al. 2011), and as expected with human pathogen, plant disease symptoms are not observed. It becomes interesting to examine how the bacteria are able to penetrate inside the plant tissue, in the absence of wounds or mechanical damage. A recent finding demonstrates clustering and penetration of *Salmonella enterica* serovar Typhimurium SL1344 (referred to as SL1344, hereafter) inside the tissues of lettuce leaf *via* stomata (Kroupitski et al. 2009). This indicates that the stomatal immunity of the plant is either not acting or is weak in the presence of *S. enterica*. However, O157:H7 infection leads to stomatal closure in Arabidopsis within 1-2 hours and the stomata remain closed for at least 8 hours (Melotto et al. 2006). Why is the stomatal response for two human pathogens different? One reason can be that SL1344 cells are able to evade recognition by the plant immune system. The other possibility is that the plant defenses acting on the pathogen are suppressed or weakened in the presence of SL1344. In either case, the response of plant cells towards infection by SL1344 seems to be different than towards infection by plant

pathogen *Pst* DC3000 and O157:H7. The virulent plant pathogen *Pst* DC3000 is able to suppress defenses and multiply vigorously in the plant, causing disease symptoms; whereas O157:H7 population does not increase and plant defenses like stomatal immunity are active (Fig. 3.1). However, with SL1344 infection, the plant is either not able to mount defense response on SL1344 infection or the signaling of immune pathways is manipulated to be suppressed. Hence, it is important to understand the plant defense mechanisms in the presence of SL1344 and modulation of which will indicate plant cell targets utilized by SL1344 for infection in the leaf tissue. Based on this understanding, the effectiveness of stomatal and apoplastic immunity of Arabidopsis against SL1344 infection was assessed and plant genome transcriptional changes caused by SL1344 infection were studied.

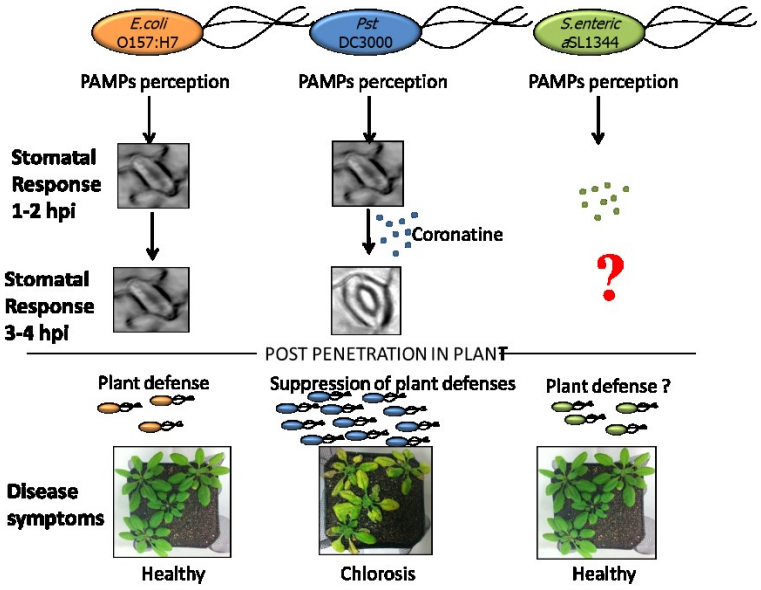

Figure 3.1 Differential response of Arabidopsis to plant pathogen and human pathogens. Perception of PAMPs leads to activation of stomatal immunity in both plant pathogen *Pst* DC3000 and human pathogen O157:H7. However, *Pst* DC3000 overcomes stomatal closure, penetrates the leaf tissue, suppresses plant defenses and multiplies in the apoplast, leading to plant symptoms. Plant defense is mounted against O157:H7 preventing bacterial multiplication. However, the action of plant defenses in SL1344 is not clear. Number of bacteria depicted is relative, and not to proportion.

3.3 Materials and Methods

3.3.1 Plant Material and Growth Conditions

Arabidopsis thaliana (L. Heyhn.) seeds were sown in a 1:1:1 v:v:v mixture of growing medium (Redi-earth plug and seedling mix, Sun Gro), fine vermiculite, and perlite. Plants were grown in controlled environmental chambers at 22^0C, 60±5% relative humidity, and a 12-h photoperiod under light intensity of 100 $\mu mol.m^{-2}.s^{-1}$. For all experiments, four- to five-week old plants were used. The ecotype Columbia (Col-0, ABRC stock CS60000) was used as the wild type plant. Hydroponically grown, naturally pest-free lettuce plants (*Lactuca sativa*, Live Gourmet, Butter Lettuce) were obtained from a local grocery store and kept at 4^0C until prior to experiments.

3.3.2 Bacterial Strains and Culturing Conditions

Wild type bacterial cells of *Escherichia coli* O157:H7 strain 86-24 and *Salmonella enterica* serovar Typhimurium SL1344 were grown in Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH= 7.0) at 30^0C for all experiments. Cells were streaked on solid medium from frozen glycerol stocks for inoculum preparation. Medium was supplemented with spectinomycin (100 $\mu g.mL^{-1}$) to grow SL1344.

3.3.3 Bacterial Inoculations

Bacterial strains were cultured at 30^0C in liquid Luria-Bertani medium supplemented with appropriate antibiotics until an OD_{600} of 0.8 to 1 was reached. Bacteria were collected by centrifugation and re-suspended in water to the final concentration of 1 x 10^8 $CFU.mL^{-1}$ containing 0.02% Silwet L-77 (Lehle Seeds Co., Round Rock, TX) for dip-inoculation of plants. Inoculated plants were immediately incubated under the following conditions: 25^0C, 60 ± 5% and 12 h of daily light (100 $\mu mol.m^{-2}.sec^{-1}$) and kept there for the

duration of the experiment. Stomatal assays were conducted as previously described (Chitrakar and Melotto, 2010) except that unstained, whole leaves were imaged with a Nikon Eclipse 80i fluorescent microscope (Nikon Corporations, Shinagawa-ku, Tokyo, Japan) equipped with long-distance objectives to measure the stomatal aperture width. For assessing internal bacterial population, leaves were surface sterilized in 70% ethanol for 2 min and bacterial population in the plant apoplast was determined as previously described (Katagiri et al. 2002).

For microarray experiment, Arabidopsis Col-0 plants were grown as described above; however to be consistent with previous microarray analysis performed by Thilmony et al. (2006), plants were acclimatized at 20^0C for one week before inoculation. *Salmonella enterica* serovar Typhimurium SL1334 were grown in Luria-Bertani medium at 30^0C and cells were harvested when the culture reached an $O.D_{600}$ of 0.8 to 1. Bacterial cells were re-suspended in sterile water to a final bacterial concentration of 1×10^8 CFU.ml^{-1} and 0.004% Silwet L-77 surfactant was added. Plants were vacuum infiltrated with the bacterial suspension and for control, plants were vacuum infiltrated with water and 0.004% Silwet L-77. Vacuum infiltration bypasses the need for stomatal opening for bacterial penetration and hence plant immunity after bacterial penetration could be assessed by this method. Plants were kept at 25^0C for 7 hours until leaves were collected for RNA extraction using TRizol® reagent (Life Technologies, Grand Island, NY) according to manufacturer's instruction. Microarray experiments were conducted in three biological replicates exactly as described by Thilmony et al. (2006).

For assays with bacterial supernatant, bacteria were grown overnight until $O.D_{600}$ of 0.8 to 1 was reached. The culture was centrifuged at 6000g for 30 minutes. The pellet was discarded and the supernatant obtained was spun again at 20,000g for 90 min (Gudesblat et al. 2009). The pellet was discarded and the supernatant was used for

experiments. Col-0 plants were dip inoculated with SL1344 culture supernatant and water (mock) using 0.02% Silwet L-77 and the stomatal assay was performed as described previously.

3.3.4 Gene Expression Analysis by Microarray

Arabidopsis Affymetrix GeneChip hybridizations were conducted in three biological replicates at Michigan State University Research Technology Support Facility exactly as described by Thilmony et al. (2006). Raw data of the biological replicates in a MIAME-compliant format is available in the Nottingham Arabidopsis Stock Centre (NASC; http://nasc.cott.ac.uk/) under the Experiment ID NASCARRAYS-674. The raw data was corrected and RMA normalized across all datasets together to obtain $\log_2$ fold change values (average of the three biological replicates). A Z-ratio-based approach was used by Dr. Bruce Rosa to identify differentially expressed genes, according to the methods outlined in Cheadle et al. (2003). This Z-ratio approach determines which genes have significantly higher fold changes than other genes in the dataset, and a Z-ratio cutoff of 2.33 was used to call approximately 2% of the genes in the dataset as being differentially expressed. Metabolism overview and biotic stress pathways were reconstructed using the Mapman software version 3.5.1 (Rotter et al. 2007). GO Single Enrichment Analysis was done using AgriGO (Zhou et al. 2010) available at http://bioinfo.cau.edu.cn/agriGO/. GO terms that were significantly (FDR-corrected $p<0.05$) more abundant in the SL1344-regulated gene, as compared to GO terms abundance in the Arabidopsis reference gene model database available at TAIR10 (Arabidopsis.org) were used for analysis.

3.3.5 Gene Expression Analysis by Real-Time PCR

Total RNA was extracted from leaves using RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL). Total RNA (1 µg) was synthesized into cDNA using the Takara RNA PCR kit (AMV) (Clontech, Montain View, CA) and diluted to a final concentration of 50 $ng.\mu L^{-1}$. Quantitative PCR (qPCR) reaction (20 µL) was performed with 10 µL of iTaq Fast SYBR Green Supermix (BioRad, Hercules, CA), 2 µL of cDNA template from the reverse transcriptase reaction described above, and 200 nM of reverse and forward gene-specific primers. Reactions were carried out in an Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA) using the following cycling parameter: 1 cycle 95^0C for 5 min and 40 cycles of 95^0C for 10 sec and 60^0C for 30 sec. *PR1* (At2g14610) gene expression level relative to the control samples were calculated using the ΔΔCt method (Livak and Schmittgen, 2001) considering the expression of the housekeeping gene *ACT8* (At1g49240) as an internal control. The primer sequences are given in Table 1. Two biological replicates and three technical replicates were performed.

3.3.6 Callose Assay

To assess the strength of PTI-triggered apoplastic immunity of Arabidopsis against SL1344 infection, a callose deposition assay was performed (Hauck et al. 2003; Nguyen et al. 2010; Adam and Somerville, 1996). Mature Arabidopsis leaves were syringe infiltrated with water (mock treatment) or 1 x 10^8 $CFU.mL^{-1}$ SL1344. After 7 hours, at least 5 leaves were harvested and chlorophyll was cleared by immersing the leaves in 95% ethanol and kept at 37^0C for 3 hours. Ethanol was replaced whenever necessary. Cleared leaves were rinsed in 50% ethanol first, and then in water. Leave were then stained for 30 min to 1 hour in 150mM K_2HPO_4 containing 0.01% aniline blue. For microscopy, leaves

were mounted on slides using 50% glycerol and imaged under DAPI filter. Infiltrated zones, damaged areas, mid vein and leaf edges were avoided for imaging to prevent false positive results. Treatments were water (mock control), *Pst* DC3000 *hrcC* mutant (positive control), *Pst* DC3000 (negative control), *E. coli* O157:H7 and *S. enterica* Typhimurium SL1344 (experimental).

3.3.7 Statistical Analysis

Statistical significance of data from the stomatal assay, bacterial counts in the apoplast, and gene expression was calculated using 2-tailed Student's *t*-test. For within group comparisons of means in callose assay, one-way ANOVA was performed and significance was measured using Tukey's HSD at $\alpha = 0.05$. All experiments reported here were repeated at least two times (biological replicates) using a minimum of three technical replicates.

3.4 Results

3.4.1 Stomatal Immunity is Compromised on SL1344 Infection.

To assess stomatal response on surface infection with SL1344, Col-0 plants were dip-inoculated with 1 x 10^8 CFU.mL^{-1} SL1344 and water (mock) and incubated at 25^0C. The abaxial leaf surface was imaged at different time points. As shown in Fig. 3.2A, it was observed that within 2 hours post infection (hpi), most of the Col-0 stomata with SL1344 infection close indicating recognition of the bacteria on the leaf surface. However, at 4 hpi, the stomata re-open and the average aperture size is similar to water control. The re-opening was observed between 3-4 hpi in every biological replicate. Similar results were obtained when the floating method was used (which allowed to monitor the same leaves over time), except that closure is very transient possibly due to higher humidity conditions

as compared to dip inoculation (Fig 3.2B). To extend the study to edible fresh produce, hydroponically grown live lettuce was used. Pieces of inner layers of lettuce leaves were floated on 1 x 10^8 CFU.mL^{-1} SL1344 bacterial suspension and water (mock) and incubated at 25^{0}C for the duration of the experiment. Similar stomatal response was seen in lettuce as well (Fig. 3.2C) with stomatal closure seen at 2 hpi and re-opening, at 4 hpi.

Lack of stomatal closure at later time points could allow for penetration of SL1344 into the leaf apoplast via the open stomata. To correlate open stomata with bacterial penetration, Col-0 plants were dip-inoculated with bacterial suspension (1 x 10^8 CFU.mL^{-1}) and water (mock) using 0.02% Silwet L-77 and the bacterial population in the plant apoplast was determined. Dip-inoculation prevents forced infiltration of bacteria into the leaf tissue and hence the bacterial numbers obtained depict the number of bacteria entered naturally into the leaf apoplast. It was observed that, at 24 h, there are approximately 8x10^3 CFU.cm^{-2} of leaf (Fig. 3.2D). However, the population drops about 100 fold in one week and remains stable for 14 days, indicating that SL1344 does not multiply vigorously in the apoplast, but is able to survive for long periods.

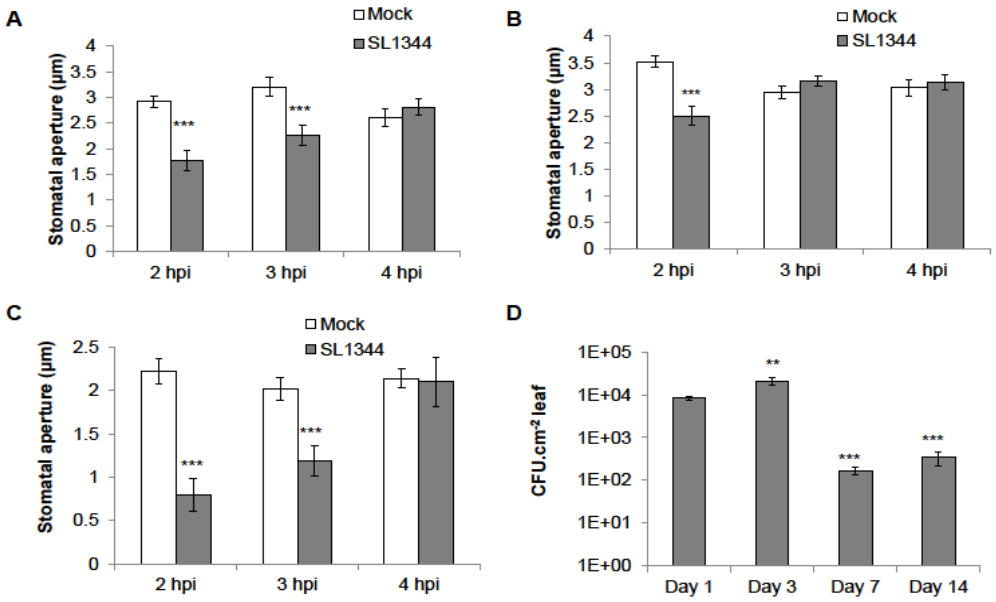

Figure 3.2 Stomatal immunity is compromised on SL1344 infection. Wild-type Col-0 plants were dipped (**A**) or leaf pieces were floated (**B**) and lettuce leaf pieces were floated (**C**), in bacterial suspensions (1 x 10^8 CFU.ml^{-1}) of SL1344 or water control (mock inoculation) and stomatal aperture width was measured 2, 3, and 4 hpi. Results are shown as the mean (n≥60) ± SE. **D,** SL1344 population count in Col-0 apoplast 1, 3, and 14 days post-inoculation. Wild-type Col-0 plants were dipped into bacterial suspensions (1 x 10^8 CFU.ml^{-1}) of SL1344 or water control (mock inoculation) and bacterial population was assessed at different time points as indicated. Results are shown as mean (n=6) ± SE. Statistical significance between the means (adjacent bars in A, B, C) was calculated with two-tailed Student's *t*-test (** = p <0.01 and *** = p <0.001).

3.4.2 Live SL1344 Bacterium is Required for Stomatal Re-Opening.

If we consider re-opening of stomata as an active mechanism of immune suppression by SL1344, then some compound must be produced by the bacterium which modulates guard cell signaling patterns leading to re-opening. Coronatine production, which re-opens stomata, is induced by *Pst* DC3000 on the leaf surface (as observed in Chapter 2) which points out to a virulence strategy required for a plant pathogen to

penetrate through the stomata and cause disease. SL1344 is not a plant pathogen and hence may not require having plant-inducible virulence strategies for infection. So, possibly a compound, which may be able to function similar to coronatine for stomatal re-opening, might be constitutively produced by SL1344 and has properties to cause stomatal opening. To test this, stomatal assays were performed as explained previously, but instead of bacterial suspension, SL1344 culture supernatant was used which would contain compounds constitutively secreted by the bacteria in growth medium. Unlike the prediction, stomatal re-opening was not observed as seen in case of live bacteria (Fig. 3.3A).

Preparation of culture supernatant required high speed centrifugation, which may have led to concentration of PAMPs in the culture supernatant. This could be responsible for strong stomatal closure (Fig. 3.3A). To check if increase in bacterium inoculum and thus PAMPs affects the stomatal response, different concentrations of SL1344 were used. It was previously reported that 1 x 10^8 CFU.mL^{-1} is the minimum dose of *Pst* DC3000 required for a robust and reproducible stomatal closure at 2 hpi and re-opening at 4 hpi (Melotto et al. 2006). Using different inoculum levels would tell us whether increase in number of bacteria, and with that, PAMPs, abolishes stomatal re-opening. As seen in Fig 3.3B, stomatal re-opening is seen in both levels of inoculum. This can indicate that some plant defenses are not activated or are overcome in the presence of high titers of live SL1344 bacteria. Although the inoculum levels may not be equivalent to concentration of PAMPs in supernatant, it is an extremely high titer for a natural infection.

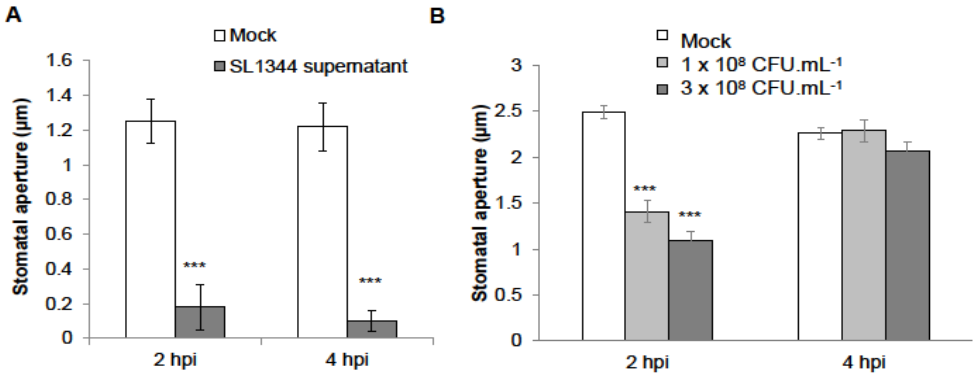

Figure 3.3 Stomatal re-opening requires the presence of live-bacteria. **A,** Col-0 plants were dipped into bacterial supernatant obtained from 1x 10^8 CFU.ml^{-1} of SL1344 or water control (mock) and stomatal aperture width was measured **B,** Col-0 plants were dipped into 1 x 10^8 CFU.ml^{-1} or 3 x 10^8 CFU.ml^{-1} of SL1344 or water (mock) and stomatal aperture width was measured. Results are shown as the mean (n≥60) ± SE. Statistical significance between the means (adjacent bars in all graphs) was calculated with two-tailed Student's *t*-test (*** = p <0.001).

Interestingly, it was observed that SL1344 supernatant induced leaf chlorosis in Arabidopsis. The "symptoms" start at the leaf margins, possibly involving guttation fluids from hydathodes, and move inward. The symptoms usually take 4-6 days to fully develop. The chlorotic symptoms are associated with significant loss of chlorophyll in the leaf as seen in Fig.3.4A. As the supernatant is diluted, its effect on the leaves is reduced with completely diminished chlorosis at 1:5 times dilution (Fig.3.4B). Another important observation was that SL1344 supernatant treated plants aged much slower than control plants (Fig. 3.5). However, it is not possible to suggest an explanation for this interesting phenomenon at this moment and is a part of an exploratory project to identify compounds secreted by SL1344 on leaf surface.

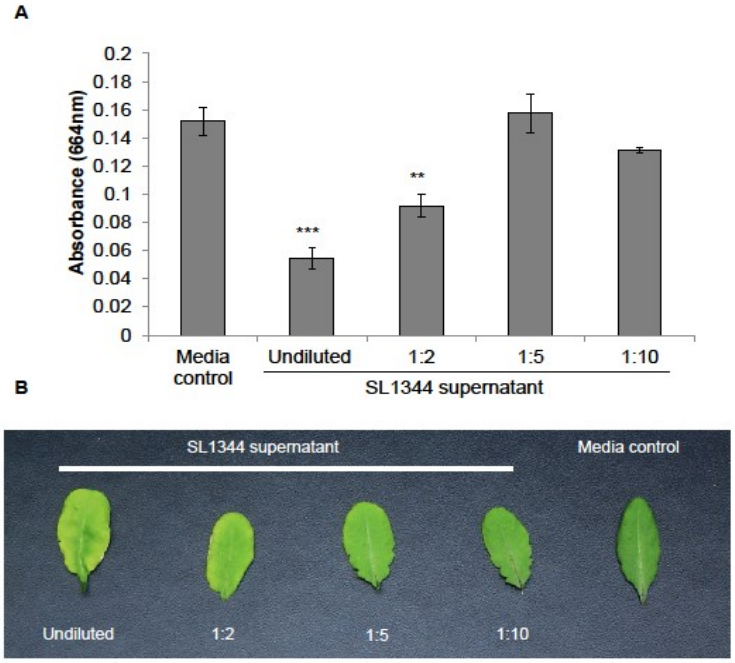

Figure 3.4 Treatment of Arabidopsis leaves with SL1344 supernatant leads to loss of chlorophyll and apparent cholorosis-like symptoms. **A,** Absorbance of chlorophyll a in leaves treated with SL1344 supernatant in the given dilutions. **B,** Chlorosis-like symptoms seen on Arabidopsis leaves treated with SL1344 supernatant in the given dilutions.

Figure 3.5 SL1344 supernatant treated plants look healthier and age slower than control (media treated) plant. Col-0 plants were treated with SL1344 supernatant or media supernatant at the age of 4 weeks and pictures were taken at the age of 6 weeks. In control, senescence indicators like anthocyanin production and darkening of leaves is seen, which is absent in SL1344 supernatant infected plants.

3.4.3 Involvement of COI1, NPR1, FLS2, GPA1 and SID2 in SL1344-Arabidopsis Interaction.

To assess if stomatal re-opening by SL1344 depends on plant signaling pathways involved in perception and hormone signaling and related to stomatal movement, homozygous Arabidopsis mutants for *COI1, NPR1, FLS2, GPA1* and *SID2* were used. COI1 and NPR1 act as receptors for jasmonic acid and salicylic acid signaling, respectively (Sheard et al. 2010, Wu et al. 2012). FLS2 is the PRR plant receptor for the bacterial PAMP, flagellin (Gómez-Gómez et al. 1999). *GPA1* encodes an *α*-subunit of the heterotrimeric G protein and is involved in abscisic acid (ABA) regulation of stomatal opening (Nilson and Assmann, 2010). *SID2* encodes an isochorismate synthase required for synthesis of SA (Wildermuth et al. 2001). When stomatal assays and bacterial inoculations were carried out in *coi1-1* and *npr1-1* mutants, it was observed that both receptors are unimportant for SL1344-trigerred closure as well as subsequent opening (Fig. 3.6A, 3.7A), as re-opening by SL1344 was seen in the mutants just like Col-0. Infection with O157:H7 also revealed no difference from the wild-type Col-0 stomatal response (Fig. 3.6B, 3.7B), suggesting that these two receptors are unimportant for general PAMP-triggered stomatal immunity. Bacterial population counts were also performed to assess the importance of jasmonic and salicylic acid signaling for bacterial survival or decline. Counts for initial days in *coi1-1* plants varied in all biological replicates performed, but a trend was observed with slightly higher counts of SL1344 in *coi1-1* plants on day 1(Fig.3.6C). However, with it was seen that SL1344 population in day 1 and 3 in *npr1-1* plants was less than Col-0 (Fig. 3.7C), suggesting that salicylic acid perception might be important for SL1344 survival in the apoplast. No difference was observed with O157:H7 (Fig. 3.7D).

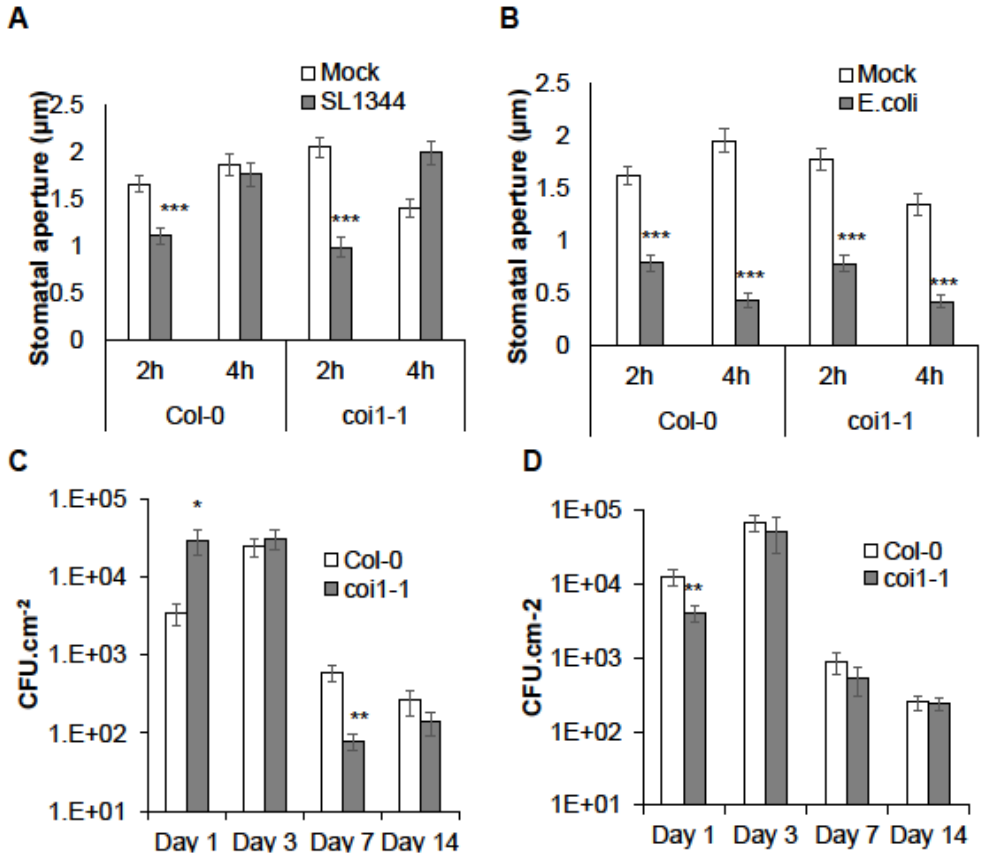

Figure 3.6 COI1 is not required for penetration and multiplication of SL1344. **A-B,** Col-0 and *coi1-1* plants were dip-inoculated with SL1344 (**A**) or *E. coli* O157:H7 (**B**) and stomatal response was assessed. Results are shown as mean of stomatal aperture width (n≥60) ± SE. Statistical significance between mock and bacterial treatment was detected with two-tailed Student's *t*-test (*** = p<0.001). **C-D,** Bacterial enumeration in the leaf apoplast at different days (d) after inoculation with SL1344 (**C**) or *E. coli* O157:H7 (**D**). Results are shown as the mean (n=18) ± SE. Statistical significance was detected with two-tailed Student's *t*-test (* = p<0.05, ** = p<0.01, *** = p<0.001).

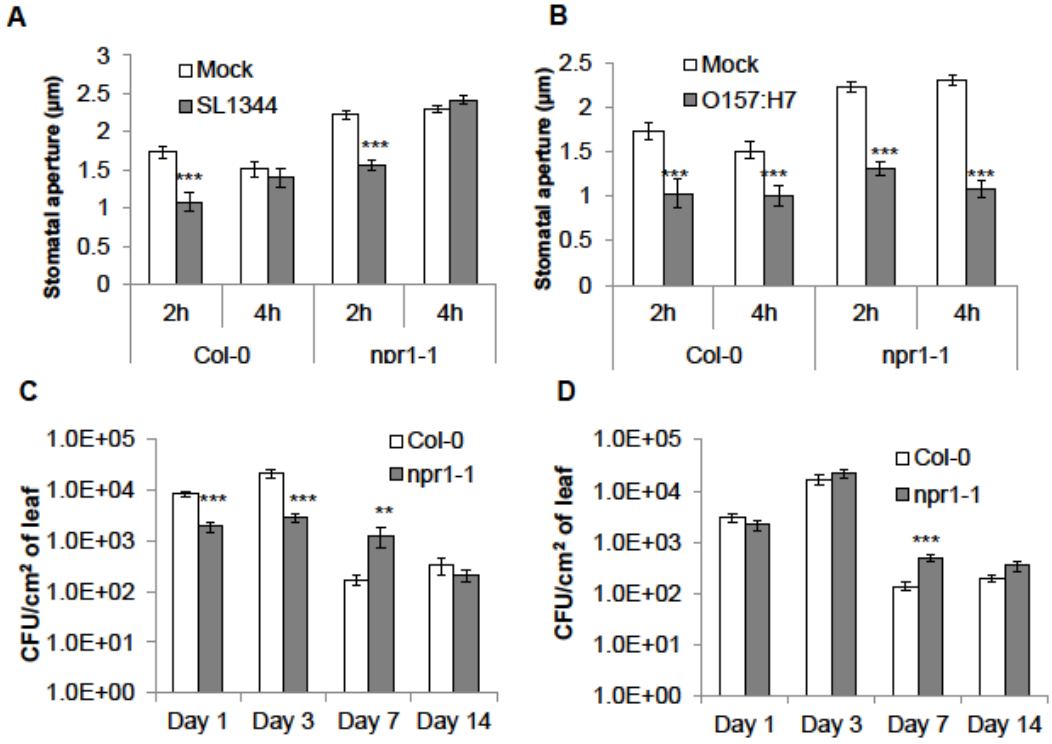

Figure 3.7 NPR1 is important for initial survival, but not penetration of SL1344. **A-B,** Col-0 and *npr1-1* plants were dip-inoculated with SL1344 (**A**) or *E. coli* O157:H7 (**B**) and stomatal response was assessed. Results are shown as mean of stomatal aperture width (n≥60) ± SE. Statistical significance between mock and bacterial treatment was detected with two-tailed Student's *t*-test (*** = p<0.001). **C-D,** Bacterial enumeration in the leaf apoplast at different days (d) after inoculation with SL1344 (**C**) or *E. coli* O157:H7 (**D**). Results are shown as the mean (n=6 or 12) ± SE. Statistical significance was detected with two-tailed Student's *t*-test (** = p<0.01, *** = p<0.001).

To observe the importance of SL1344 flagellin perception for stomatal response, *fls2* mutant plants were used. As shown in Fig. 3.8A, SL1344-triggered stomatal closure is delayed in *fls2* mutant plants, and stomatal re-opening is not observed at 4 h, as observed in wild type plant Col-0. This experiment was performed once, but two mutant lines for FLS2 were used for confirmation and similar results were obtained. Response to O157:H7 is similar to wild-type, in that stomatal closure is seen within 2 h (Fig.3.8B). This suggests

that SL1344 recognition is delayed in the absence of FLS2, and FLS2 may be needed for stomatal re-opening in the presence of SL1344. Population counts inside leaf apoplast was estimated at early time points till day 3 since flagellin perception, being part of PTI, should occur very early in the infection process. It was observed that both bacteria grow better in *fls2* mutant plant at later stages of infection, as seen by higher counts on day 3 post infection (Fig. 3.8C, D), suggesting that FLS2 or some part of its signaling may be involved in resistance in the apoplast. Slightly lower counts in *fls2* are also seen for both bacteria in the first day.

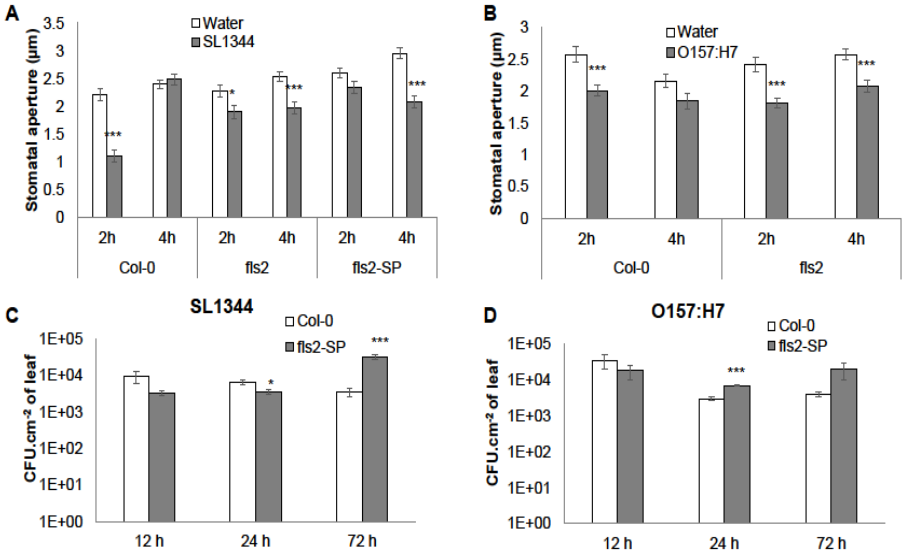

Figure 3.8 Stomatal response and bacterial counts in *fls2* plants. **A-B,** Col-0 and *fls2* plants were dip-inoculated with SL1344 (**A**) or *E. coli* O157:H7 (**B**) and stomatal response was assessed. Results are shown as mean of stomatal aperture width (n≥60) ± SE. **C-D,** Bacterial enumeration in the leaf apoplast at different hours (h) after inoculation with SL1344 (**C**) or *E. coli* O157:H7 (**D**). Results are shown as the mean (n=6) ± SE. Statistical significance was detected with two-tailed Student's *t*-test (* = p<0.05, ** = p<0.01, *** = p<0.001).

Stomatal response to SL1344 and O157:H7 was also assessed in *gpa1* and *sid2* mutants. As shown in Fig. 3.9, stomatal closure and subsequent re-opening is seen in both mutants in response to SL1344, and constant stomatal closure is seen in response to O157:H7 for 4 h. This is similar to wild-type plant response. Only one biological replicate was performed in this experiment, but the results have been validated by another student continuing this project. Thus, it can be suggested that GPA1 and SID2 proteins are not involved in stomatal movement in the presence of human pathogens.

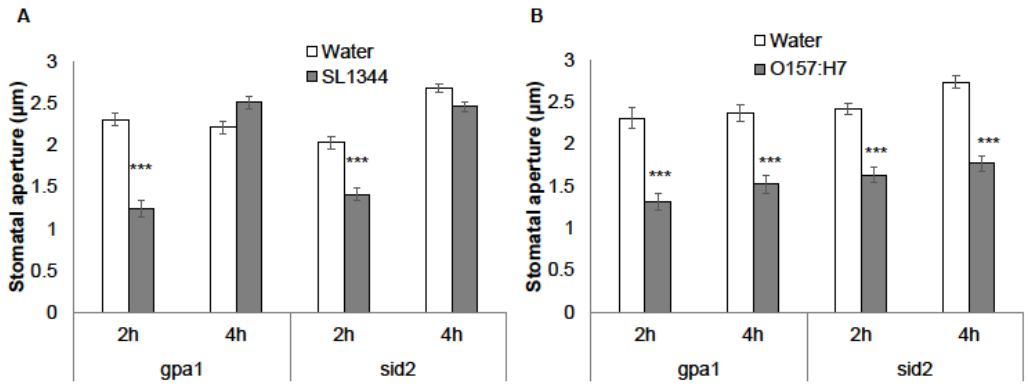

Figure 3.9 Stomatal response and bacterial counts in *gpa1* and *sid2* plants. Mutant plants were dip-inoculated with SL1344 (**A**) or *E. coli* O157:H7 (**B**) and stomatal response was assessed. Results are shown as mean of stomatal aperture width (n≥60) ± SE. Statistical significance was detected with two-tailed Student's t-test (*** = p<0.001).

3.4.4 Salicylic Acid Defense Signaling is Weaker in SL1344 as Compared to E. coli O157:H7.

To assess if the plant immunity against SL1344 is weak, expression of a defense marker gene *PR1* was checked in Col-0 plants surface-inoculated with 1 x 10^8 CFU.mL^{-1} inoculum of SL1344 or water. Infection with *E. coli* O157:H7 was carried out for comparison of defense response. Leaf tissue was collected from each treatment at different time points and was flash frozen. This frozen tissue was used for RNA extraction and subsequent

cDNA construction as described previously. Quantitative PCR was performed as described previously. As shown in Fig.3.10, both *E. coli* O157:H7 and SL1344 induce an early expression of *PR1* gene; however O157:H7 induces a higher level of *PR1* than SL1344. This result suggests that the plant may activate its defense more effectively against O157:H7 than SL1344. However, the signal for this SA-based defense diminishes after 24 h and this suggests that salicylic acid signaling might be important in early defenses against human pathogens.

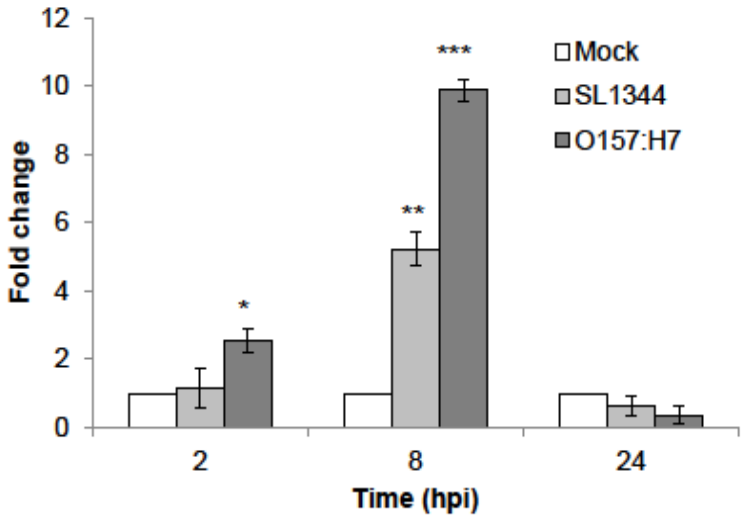

Figure 3.10 O157:H7 induces higher level of *PR1* gene expression than SL1344. Expression of *PR1* gene at 2 h, 8 h and 24 h post inoculation with bacteria relative to the mock control was determined by qPCR. Statistical significance of the difference in the mean expression of *PR1* in response to SL1344 and O157:H7 relative to that in the mock control was detected with two-tailed Student's *t*-test (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

3.4.5 Apoplastic Immunity is Compromised in the Presence of SL1344.

During early stages of pathogen infection, callose-containing cell-wall appositions are formed. These make effective barriers that are induced at the sites of attack. Callose is an amorphous, high–molecular weight β-(1,3)-glucan polymer that serves as a matrix in which antimicrobial compounds can be deposited, thereby providing focused delivery of chemical defenses at the cellular sites of attack. Callose deposition is typically triggered by conserved pathogen-associated molecular patterns (PAMPs) (Brown et al. 1998; Gomez-Gomez et al. 1999) and is being steadily used as a marker for PTI (Luna et al. 2011). To assess the strength of PTI-triggered apoplastic immunity of Arabidopsis against SL1344 infection, a callose deposition assay was performed. Along with water, *Pst* DC3000 was used as a negative control as it is able to suppress callose depositions and *Pst* DC3000 *hrcC*$^{-}$ mutant was used positive control as this avirulent bacterium induces maximum callose deposition (Hauck et al. 2003). Mature Arabidopsis leaves were pressure infiltrated with water or 1 x 10^{8} CFU.mL^{-1} bacteria and callose deposition was viewed using aniline blue staining. As shown in Fig 3.11, the positive control *hrcC* mutant induced a large number of callose deposits in Col-0 leaves. A significantly lower number of deposits were seen in leaves infected with *Pst* DC3000 as expected. With human pathogens, it was found that SL1344 also caused lower number of callose deposits, indicating that callose-associated cell wall defense is compromised in the presence of SL1344. *E. coli* O157:H7 infection induced very high levels of callose deposits, similar to the positive control. Interestingly, statistical analysis no difference between water, SL1344, and *Pst* DC3000 treatments, indicating that either SL1344 is not even recognized as a potential invader (like water) or it is able to suppress callose deposition (like virulent plant pathogen *Pst* DC3000).

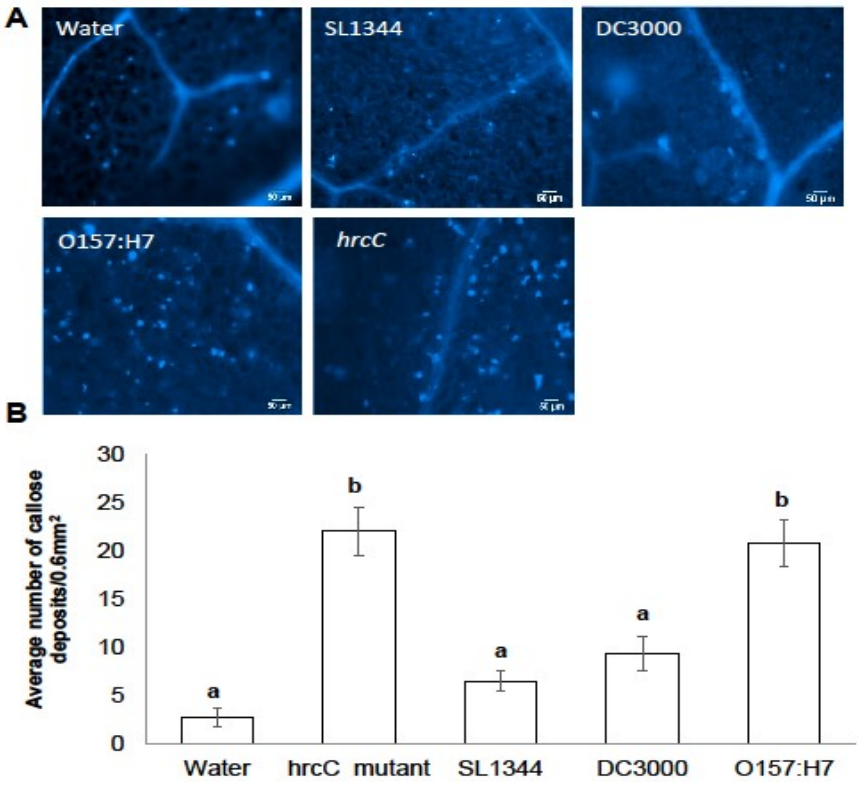

Figure 3.11 Callose deposition is compromised by SL1344 infection. Col-0 leaves were pressure infiltrated with 1 x 10^8 CFU.mL^{-1} of bacteria or water and callose was observed after 7 h. **A,** Representative micrographs of leaf surfaces stained with aniline blue for callose deposits. Blue dots in the figure are callose deposits. **B,** Average number of callose deposits from two independent experiments (n=32). One-way ANOVA revealed statistical differences between treatments ($F = 21.3276$; $p<0.001$; $df = 4$). Different letters indicate statistically significant differences between treatments (Tukey's HSD test; $\alpha = 0.05$).

3.4.6 Gene Expression in Whole Leaves Infected with SL1344.

A genome-wide microarray can not only provide clues about how PAMP-mediated stomatal closure is inhibited, but also a comprehensive knowledge about differential response of Arabidopsis leaf cells towards different pathogens. Hence, a microarray experiment was conducted to evaluate gene expression changes in Col-0 leaves on infection with SL1344.

Col-0 leaves were vacuum-infiltrated with water (mock) or bacterial suspension using 0.004% Silwet L-77 and plants were incubated at 20^{o}C for 7 hours. Total RNA from 25 Col-0 leaves was extracted. cDNA preparation and Arabidopsis ATH1 GeneChip array hybridization was performed at Michigan State University Genomics Facility, according to Thilmony et al. (2006). A total of 10 genes were chosen for gene expression validation by RT-qPCR. Seven genes of the ten were confirmed by another student, Dr. Giselle Carvalho, and all genes are shown in Fig. 3.12. All genes showed that same expression pattern in both RT-qPCR and microarray.

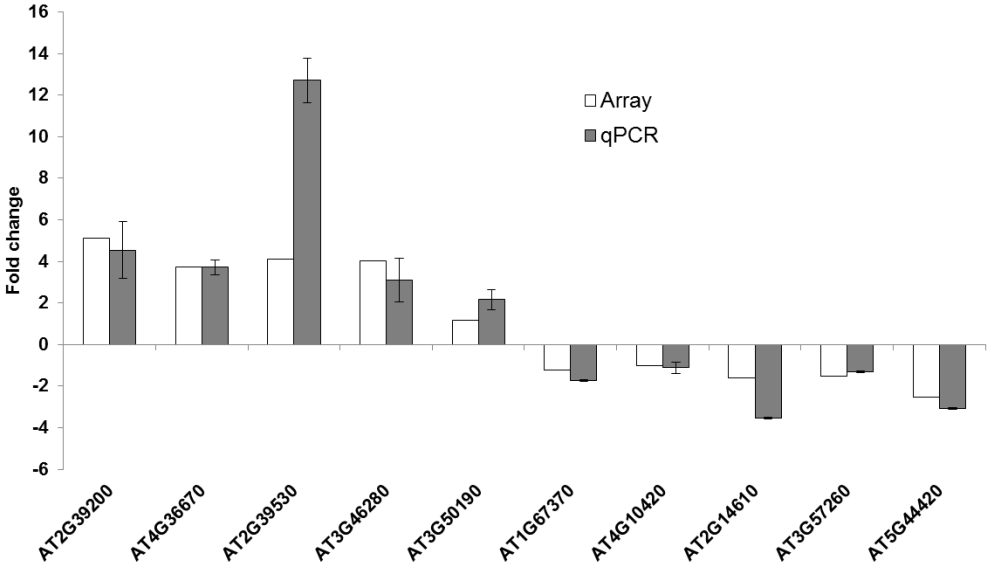

Figure 3.12 Comparison of fold change values obtained from microarray and qPCR experiments.

To assess the commonalities in transcriptional changes in leaves treated with SL1344 and other bacteria, previously published data for *E. coli* O157:H7 and *Pst* DC3000 (Thilmony et al. 2006) were used. Genes that were significant in differentially regulated by any bacterial treatment over mock as indicated by Z-ratio analysis were used. As shown in Fig. 3.13, a total of 45 genes are commonly regulated by all three bacteria, indicating a

generalized plant response to bacterial infection. The maximum number of commonalities are seen between the two human pathogens, where a total of 150 genes are commonly regulated in the presence of both human pathogens. Of this, 123 genes are up-regulated, indicating a generic plant response to human pathogens. The number of genes down-regulated by both O157:H7 and *Pst* DC3000 is more than those shared between S1344 and *Pst* DC3000, indicating that these genes may be modulated differentially by SL1344. There were only 20 genes commonly down-regulated in response to *Pst* DC3000 and SL1344. Of those genes, two are WRKY transcription factors (WRKY33 and WRKY53) coding genes. WRKYs are known to be involved in antagonistic hormonal defense signaling in Arabidopsis. Since SL1344 infection also downregulates these genes, it is possible that SL1344 modulates some component of hormonal signaling. SA signaling is one prospective pathway to be explored in SL1344 infection since lesser *PR1* gene induction and requirement of SA signaling (Fig. 3.10) for survival in the apoplast is observed (Fig. 3.7). AtWRKY33 functions as a positive regulator of resistance toward the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* (Zheng et al. 2006), and AtWRKY53 and positively modulates SAR (Wang et al. 2006). Moreover, SA biosynthesis and expression of *NPR1*, a key central regulator of SA-dependent defenses and SAR, also appear to be regulated by WRKY TFs (Yu et al., 2001).

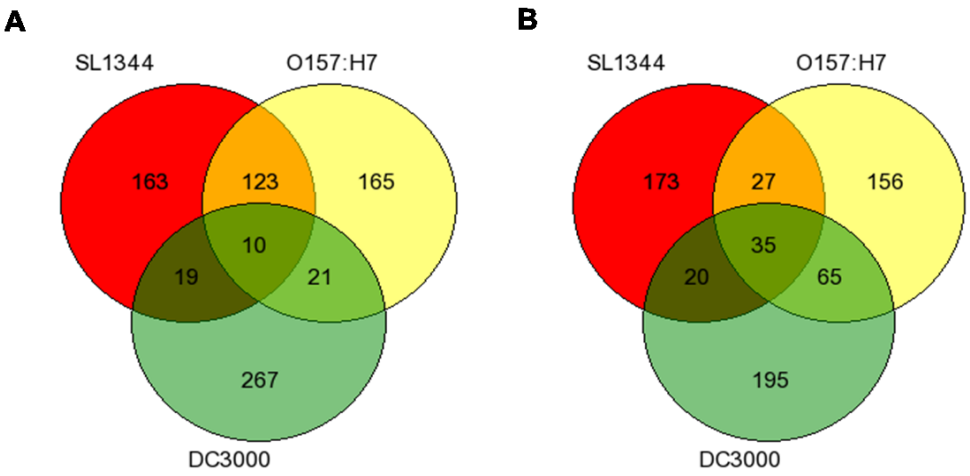

Figure 3.13 Shared differentially regulated genes between datasets. (A) Up-regulated genes between the SL1344, O157:H7 and DC3000 datasets. (B) Down-regulated genes between the SL1344, O157:H7 and DC3000 datasets.

3.4.7 Mapman Overview of Pathways Related to Biotic Stress

The differentially regulated gene set as determined by the Z-ratio analysis (SL1344 vs. mock-treated plants) was classified using Mapman platform to obtain an overview of the predicted gene functions related to the pathogen or biotic stress in Arabidopsis. There is a strong indication of differential involvement of auxin, ethylene, jasmonate, and abscisic acid hormone signaling as shown in Fig.3.14. Each box represents a single gene in the figure. The dark shaded area represents data supported by experiments and the light area represents putative involvement. Signaling pathways, involving several genes coding for calcium binding protein, protein kinases, and calmodulin binding proteins are significantly regulated. Two of the genes in this list of signaling pathways are PAMP receptors. FRK1 (Flg22-induced receptor like kinase1), which is activated by the well-known PAMP, flagellin and CERK1 (Chitin Elicitor receptor kinase), perceives fungal chitin. Both these genes are up-regulated in response to SL1344. The two genes in the dark shaded area are both

related to defense and they are *MLO12* (Mildew Resistance Locus O 12) and a defensin like protein. Among the plant hormones, ABA, auxin, jasmonate, and ethylene are seen to be involved. In jasmonate signaling, surpisingly, JA biosynthesis genes *LOX2* and *OPR3* are differently regulated. All bins have both positively and negatively regulated gene members, suggesting differential regulation of pathway members by SL1344. Several ubiquitin related and F-box proteins are differentially regulated by SL1344. Genes involved in secondary metabolism are also up-regulated. Production of low-molecular weight compounds, known as secondary metabolites, is a common defense strategy of plants (Bednarek, 2012). Cell wall modification proteins were also significantly up-regulated. One of these genes, *AtEXPA1,* is of interest with respect to stomatal immunity. This gene codes for an expansin which regulates stomatal movement by cell wall modifications of the guard cell. Overexpression of this gene increases the rate of light-induced stomatal opening (Wei et al. 2011). *AtEXPA1* is also connected to the hormone auxin, which was previously mentioned as differentially regulated by SL1344 treatment. The connection between auxin and *AtEXPA1* is that auxin signaling activates expansins using H^+ATPase. It is through this that *AtEXPA1* modulates stomatal movement. A marker gene for defense *PR2* is seen to be down-regulated by SL1344 treatment.

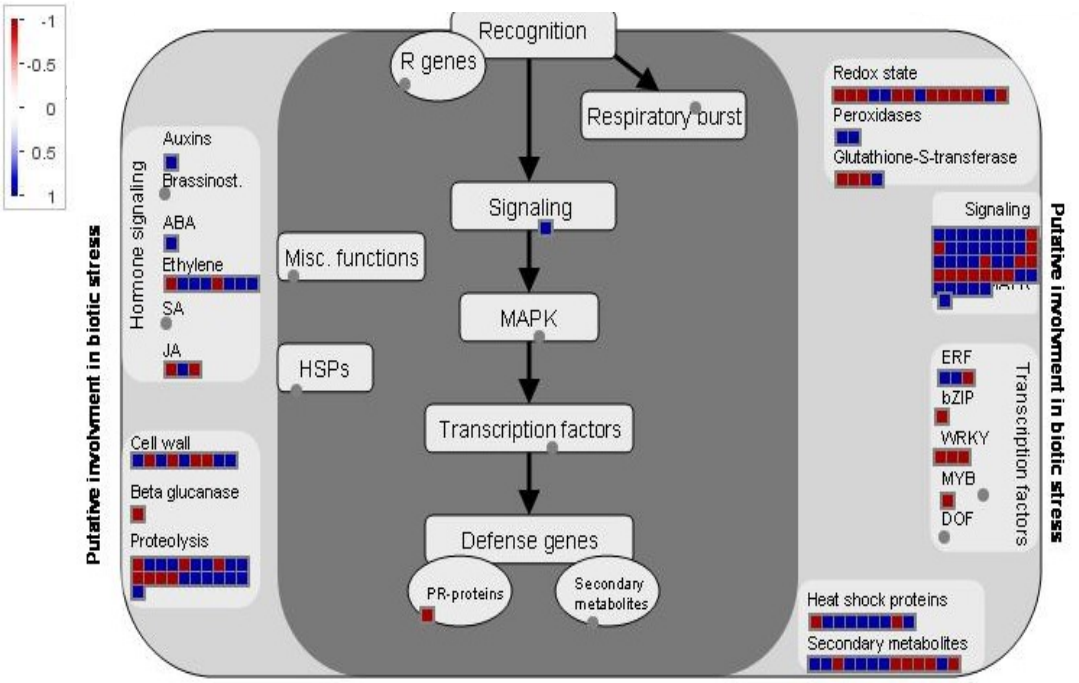

Figure 3.14 Mapman overview of genes putatively involved in biotic stress, created by using Col-0 genes differentially regulated by SL1344 infection.

3.4.8 Gene Ontology Analysis Reveals Over-Representation of Several GO Categories

GO enrichment analysis (Table 2) revealed that 196 GO terms are significantly (FDR-corrected $p<0.05$) more abundant in the SL1344-regulated gene lists, as compared to GO terms abundance in the Arabidopsis reference gene model database available at TAIR10 (Arabidopsis.org). From Fig. 3.15, it is observed that many GOs are over-represented by SL1344 gene regulation. Here, the percentage for the input list is calculated by the number of genes mapped to the GO term divided by the number of all genes in the input list. The same calculation was applied to the reference list to generate its percentage. These two lists are represented using different custom colors. Among the GO for Biological Process (P), metabolic processes, cellular process, multi organism process, response to stimulus are over-represented. Cellular Component (C) GOs like cell part, extracellular

region and organelle, and molecular function (F) GOs like catalytic activity, transporter activity and structural molecule activity are also have more percent of genes in the input list.

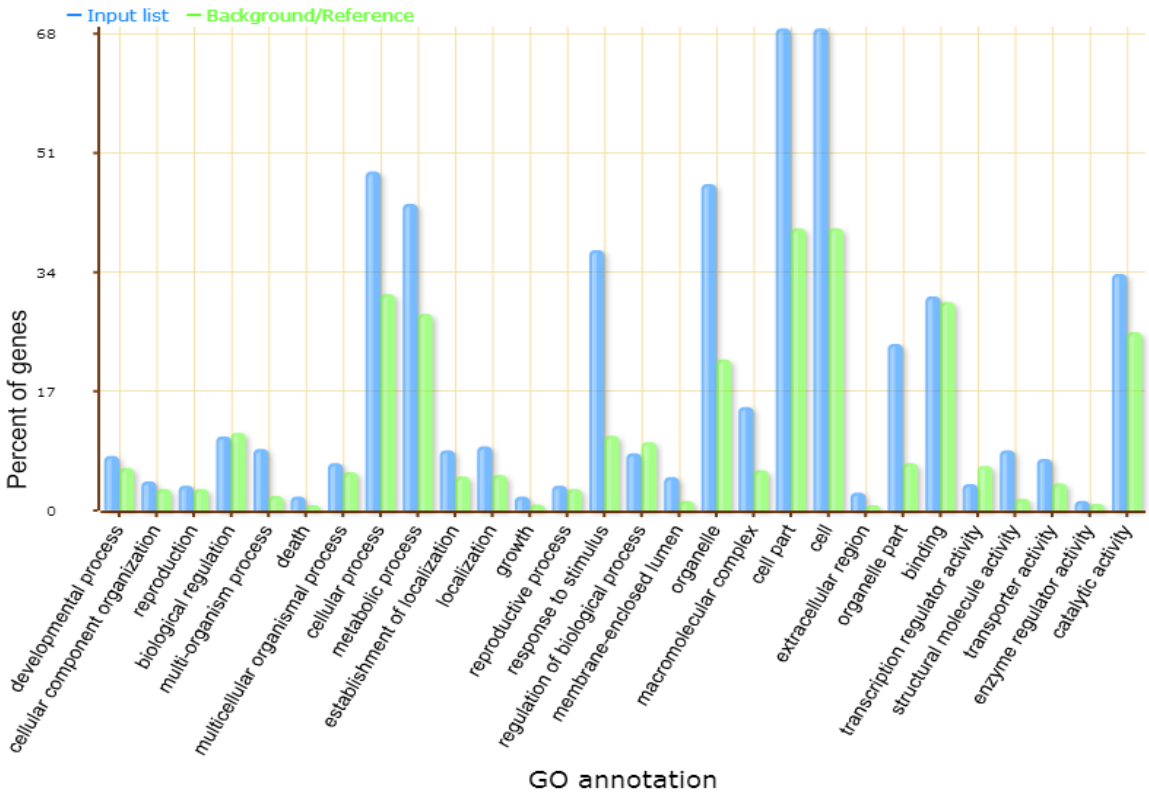

Figure 3.15 Flash bar chart of overrepresented terms in all three categories. The input list is the list of genes that are significantly regulated in response to SL1344 as obtained from Z-ratio analysis. The Y-axis is the percentage of genes mapped by the term, and represents the abundance of the GO term. The X-axis is the definition of GO terms.

Table 2 GO Single Enrichment Analysis (SEA) of genes significantly regulated in response to SL1344 infection. Statistical significance was calculated using Fisher Exact test ($p<0.05$) with Hochberg correction (FDR). GO terms are Biological Process (P), Molecular Function (F), and Cellular Component (C).

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0050896	P	response to stimulus	200	4057	1.80E-59	5.10E-57
GO:0006950	P	response to stress	136	2320	3.00E-46	8.80E-44
GO:0042221	P	response to chemical stimulus	119	2085	5.50E-39	1.60E-36
GO:0009628	P	response to abiotic stimulus	92	1471	1.20E-32	3.40E-30
GO:0009607	P	response to biotic stimulus	53	638	3.90E-24	1.10E-21
GO:0009266	P	response to temperature stimulus	46	485	3.10E-23	9.10E-21
GO:0051707	P	response to other organism	50	599	6.70E-23	1.90E-20
GO:0015979	P	photosynthesis	28	162	1.00E-20	2.90E-18
GO:0051704	P	multi-organism process	52	776	8.10E-20	2.30E-17
GO:0009409	P	response to cold	34	328	1.50E-18	4.40E-16
GO:0010033	P	response to organic substance	66	1342	3.10E-18	8.90E-16
GO:0010035	P	response to inorganic substance	31	279	8.50E-18	2.50E-15
GO:0044237	P	cellular metabolic process	209	8722	1.10E-17	3.30E-15
GO:0034641	P	cellular nitrogen compound metabolic process	39	506	5.30E-17	1.50E-14
GO:0009611	P	response to wounding	26	197	9.20E-17	2.70E-14

Table 2 continued.

GO:0009605	P	response to external stimulus	35	429	4.50E-16	1.30E-13
GO:0006952	P	defense response	46	766	5.90E-16	1.70E-13
GO:0009987	P	cellular process	249	11684	2.00E-15	5.80E-13
GO:0008152	P	metabolic process	228	10614	4.50E-14	1.30E-11
GO:0009617	P	response to bacterium	25	247	9.20E-14	2.70E-11
GO:0043436	P	oxoacid metabolic process	44	859	4.90E-13	1.40E-10
GO:0019752	P	carboxylic acid metabolic process	44	859	4.90E-13	1.40E-10
GO:0006082	P	organic acid metabolic process	44	860	5.10E-13	1.50E-10
GO:0009620	P	response to fungus	20	158	6.70E-13	1.90E-10
GO:0042180	P	cellular ketone metabolic process	44	882	1.10E-12	3.30E-10
GO:0006091	P	generation of precursor metabolites and energy	25	285	1.70E-12	5.00E-10
GO:0009743	P	response to carbohydrate stimulus	23	240	2.60E-12	7.40E-10
GO:0006979	P	response to oxidative stress	26	332	6.70E-12	1.90E-09
GO:0009414	P	response to water deprivation	22	229	7.30E-12	2.10E-09
GO:0009415	P	response to water	22	240	1.70E-11	4.90E-09
GO:0009416	P	response to light stimulus	33	596	6.60E-11	1.90E-08
GO:0006970	P	response to osmotic stress	27	408	9.50E-11	2.70E-08
GO:0009314	P	response to radiation	33	613	1.30E-10	3.80E-08
GO:0010200	P	response to chitin	17	151	1.90E-10	5.50E-08
GO:0009651	P	response to salt stress	25	366	2.50E-10	7.40E-08
GO:0006725	P	cellular aromatic compound metabolic process	26	399	2.90E-10	8.30E-08

Table 2 continued.

GO:0010876	P	lipid localization	9	24	4.50E-10	1.30E-07
GO:0006519	P	cellular amino acid and derivative metabolic process	34	682	4.70E-10	1.30E-07
GO:0009058	P	biosynthetic process	123	5118	7.60E-10	2.20E-07
GO:0019438	P	aromatic compound biosynthetic process	19	237	3.20E-09	9.40E-07
GO:0019748	P	secondary metabolic process	27	489	3.90E-09	1.10E-06
GO:0044249	P	cellular biosynthetic process	117	4925	4.40E-09	1.30E-06
GO:0044271	P	cellular nitrogen compound biosynthetic process	24	394	4.90E-09	1.40E-06
GO:0006790	P	sulfur metabolic process	18	220	6.30E-09	1.80E-06
GO:0019684	P	photosynthesis, light reaction	13	103	7.60E-09	2.20E-06
GO:0009719	P	response to endogenous stimulus	41	1068	1.20E-08	3.30E-06
GO:0042742	P	defense response to bacterium	16	177	1.20E-08	3.40E-06
GO:0050832	P	defense response to fungus	13	108	1.30E-08	3.70E-06
GO:0016053	P	organic acid biosynthetic process	24	417	1.40E-08	3.90E-06
GO:0046394	P	carboxylic acid biosynthetic process	24	417	1.40E-08	3.90E-06
GO:0042398	P	cellular amino acid derivative biosynthetic process	18	233	1.40E-08	4.10E-06
GO:0009408	P	response to heat	15	161	2.30E-08	6.80E-06
GO:0006520	P	cellular amino acid metabolic process	24	430	2.40E-08	6.90E-06
GO:0044272	P	sulfur compound biosynthetic process	13	115	2.50E-08	7.30E-06
GO:0044106	P	cellular amine metabolic process	24	438	3.30E-08	9.50E-06

Table 2 continued.

GO:0044248	P	cellular catabolic process	32	746	4.50E-08	1.30E-05
GO:0006575	P	cellular amino acid derivative metabolic process	20	315	4.90E-08	1.40E-05
GO:0000302	P	response to reactive oxygen species	11	85	8.60E-08	2.50E-05
GO:0010038	P	response to metal ion	17	238	1.00E-07	3.00E-05
GO:0009642	P	response to light intensity	11	90	1.50E-07	4.20E-05
GO:0009308	P	amine metabolic process	25	521	1.90E-07	5.50E-05
GO:0009725	P	response to hormone stimulus	36	982	2.70E-07	7.80E-05
GO:0019253	P	reductive pentose-phosphate cycle	5	7	3.40E-07	9.90E-05
GO:0046686	P	response to cadmium ion	14	178	4.70E-07	0.00014
GO:0019685	P	photosynthesis, dark reaction	5	8	5.50E-07	0.00016
GO:0042254	P	ribosome biogenesis	16	241	6.20E-07	0.00018
GO:0022613	P	ribonucleoprotein complex biogenesis	16	253	1.10E-06	0.00033
GO:0044267	P	cellular protein metabolic process	83	3487	1.20E-06	0.00034
GO:0034614	P	cellular response to reactive oxygen species	5	10	1.30E-06	0.00036
GO:0034599	P	cellular response to oxidative stress	5	11	1.80E-06	0.00052
GO:0008652	P	cellular amino acid biosynthetic process	14	202	1.90E-06	0.00056
GO:0044238	P	primary metabolic process	172	8995	1.90E-06	0.00056
GO:0009644	P	response to high light intensity	8	57	2.90E-06	0.00085

Table 2 continued.

GO:0006412	P	translation	43	1445	4.00E-06	0.0011
GO:0000096	P	sulfur amino acid metabolic process	9	84	5.40E-06	0.0016
GO:0006955	P	immune response	18	367	7.40E-06	0.0021
GO:0009309	P	amine biosynthetic process	14	229	7.50E-06	0.0022
GO:0009767	P	photosynthetic electron transport chain	7	46	7.60E-06	0.0022
GO:0002376	P	immune system process	18	368	7.70E-06	0.0022
GO:0015977	P	carbon fixation	5	16	7.90E-06	0.0023
GO:0051179	P	localization	51	1922	1.10E-05	0.0032
GO:0019538	P	protein metabolic process	88	4009	1.30E-05	0.0037
GO:0046483	P	heterocycle metabolic process	20	460	1.30E-05	0.0037
GO:0042434	P	indole derivative metabolic process	7	53	1.80E-05	0.0051
GO:0042430	P	indole and derivative metabolic process	7	53	1.80E-05	0.0051
GO:0044275	P	cellular carbohydrate catabolic process	10	125	1.80E-05	0.0051
GO:0033554	P	cellular response to stress	18	399	2.20E-05	0.0062
GO:0016052	P	carbohydrate catabolic process	10	128	2.20E-05	0.0062
GO:0070887	P	cellular response to chemical stimulus	19	452	3.20E-05	0.0094
GO:0006810	P	transport	48	1846	3.30E-05	0.0097
GO:0051234	P	establishment of localization	48	1851	3.60E-05	0.01
GO:0006007	P	glucose catabolic process	8	83	3.60E-05	0.01

Table 2 continued.

GO:0009737	P	response to abscisic acid stimulus	17	378	3.80E-05	0.011
GO:0019320	P	hexose catabolic process	8	84	3.90E-05	0.011
GO:0046365	P	monosaccharide catabolic process	8	84	3.90E-05	0.011
GO:0033036	P	macromolecule localization	19	462	4.30E-05	0.012
GO:0006006	P	glucose metabolic process	8	86	4.60E-05	0.013
GO:0009814	P	defense response, incompatible interaction	10	143	5.20E-05	0.015
GO:0022900	P	electron transport chain	8	88	5.30E-05	0.015
GO:0046164	P	alcohol catabolic process	8	89	5.70E-05	0.017
GO:0051716	P	cellular response to stimulus	27	840	7.60E-05	0.022
GO:0044085	P	cellular component biogenesis	21	571	8.10E-05	0.023
GO:0042435	P	indole derivative biosynthetic process	6	47	8.40E-05	0.024
GO:0032787	P	monocarboxylic acid metabolic process	17	408	9.20E-05	0.027
GO:0009695	P	jasmonic acid biosynthetic process	5	29	9.40E-05	0.027
GO:0055086	P	nucleobase, nucleoside and nucleotide metabolic process	12	221	9.80E-05	0.028
GO:0000097	P	sulfur amino acid biosynthetic process	6	49	0.0001	0.03
GO:0019318	P	hexose metabolic process	9	126	0.00011	0.03
GO:0031408	P	oxylipin biosynthetic process	5	32	0.00014	0.041
GO:0042542	P	response to hydrogen peroxide	6	53	0.00015	0.045
GO:0045087	P	innate immune response	15	347	0.00016	0.046

Table 2 continued.

GO:0042538	P	hyperosmotic salinity response	6	54	0.00017	0.049
GO:0003735	F	structural constituent of ribosome	39	494	2.50E-17	2.40E-15
GO:0005198	F	structural molecule activity	44	659	7.20E-17	6.80E-15
GO:0016491	F	oxidoreductase activity	50	1463	1.10E-08	9.90E-07
GO:0008943	F	glyceraldehyde-3-phosphate dehydrogenase activity	5	12	2.50E-06	0.00024
GO:0016829	F	lyase activity	19	430	1.70E-05	0.0016
GO:0003824	F	catalytic activity	176	9638	2.40E-05	0.0022
GO:0016620	F	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	7	56	2.40E-05	0.0023
GO:0016168	F	chlorophyll binding	6	38	2.90E-05	0.0027
GO:0016903	F	oxidoreductase activity, acting on the aldehyde or oxo group of donors	8	84	3.90E-05	0.0037
GO:0016209	F	antioxidant activity	10	150	7.60E-05	0.0071
GO:0005509	F	calcium ion binding	12	215	7.70E-05	0.0072
GO:0022892	F	substrate-specific transporter activity	31	1059	0.00012	0.011
GO:0005215	F	transporter activity	39	1477	0.00014	0.013
GO:0022857	F	transmembrane transporter activity	31	1128	0.00034	0.032
GO:0016765	F	transferase activity, transferring alkyl or aryl (other than methyl) groups	9	158	0.00051	0.048
GO:0005737	C	cytoplasm	242	6822	1.40E-48	1.30E-46

Table 2 continued.

GO:0044444	C	cytoplasmic part	228	6289	1.60E-46	1.50E-44
GO:0044424	C	intracellular part	272	9302	1.70E-40	1.60E-38
GO:0005622	C	intracellular	275	9671	7.50E-39	6.90E-37
GO:0044464	C	cell part	357	15217	1.20E-37	1.10E-35
GO:0005623	C	cell	357	15217	1.20E-37	1.10E-35
GO:0043229	C	intracellular organelle	238	8149	1.50E-33	1.40E-31
GO:0043226	C	organelle	238	8155	1.70E-33	1.50E-31
GO:0044446	C	intracellular organelle part	121	2561	3.80E-32	3.60E-30
GO:0044422	C	organelle part	121	2562	4.00E-32	3.70E-30
GO:0043231	C	intracellular membrane-bounded organelle	216	7615	8.30E-28	7.70E-26
GO:0043227	C	membrane-bounded organelle	216	7622	9.40E-28	8.70E-26
GO:0016020	C	membrane	144	4068	5.90E-26	5.50E-24
GO:0044435	C	plastid part	62	867	7.20E-25	6.70E-23
GO:0009536	C	plastid	117	2965	1.80E-24	1.70E-22
GO:0034357	C	photosynthetic membrane	36	273	1.00E-22	9.60E-21
GO:0009579	C	thylakoid	40	376	5.50E-22	5.10E-20
GO:0044434	C	chloroplast part	54	746	5.60E-22	5.20E-20
GO:0009507	C	chloroplast	107	2740	5.90E-22	5.40E-20
GO:0005829	C	cytosol	56	912	1.30E-19	1.20E-17
GO:0022626	C	cytosolic ribosome	34	336	3.00E-18	2.80E-16
GO:0009521	C	photosystem	19	66	5.00E-18	4.70E-16
GO:0005840	C	ribosome	39	524	1.60E-16	1.50E-14

Table 2 continued.

GO:0009535	C	chloroplast thylakoid membrane	26	231	3.00E-15	2.70E-13
GO:0055035	C	plastid thylakoid membrane	26	231	3.00E-15	2.70E-13
GO:0042651	C	thylakoid membrane	26	244	9.70E-15	9.00E-13
GO:0044436	C	thylakoid part	28	307	3.20E-14	3.00E-12
GO:0033279	C	ribosomal subunit	31	389	4.00E-14	3.70E-12
GO:0032991	C	macromolecular complex	78	2180	4.80E-14	4.50E-12
GO:0009534	C	chloroplast thylakoid	27	290	5.90E-14	5.50E-12
GO:0031090	C	organelle membrane	45	842	6.50E-14	6.00E-12
GO:0031976	C	plastid thylakoid	27	293	7.50E-14	6.90E-12
GO:0031984	C	organelle subcompartment	27	295	8.70E-14	8.10E-12
GO:0043232	C	intracellular non-membrane-bounded organelle	50	1040	1.10E-13	1.10E-11
GO:0043228	C	non-membrane-bounded organelle	50	1040	1.10E-13	1.10E-11
GO:0044445	C	cytosolic part	29	360	2.10E-13	1.90E-11
GO:0022625	C	cytosolic large ribosomal subunit	20	162	1.00E-12	9.50E-11
GO:0030529	C	ribonucleoprotein complex	39	703	1.10E-12	1.00E-10
GO:0005618	C	cell wall	29	403	2.80E-12	2.60E-10
GO:0030312	C	external encapsulating structure	29	407	3.50E-12	3.30E-10
GO:0005773	C	vacuole	28	383	4.80E-12	4.40E-10
GO:0015934	C	large ribosomal subunit	21	225	3.60E-11	3.40E-09
GO:0005886	C	plasma membrane	55	1456	5.60E-11	5.20E-09
GO:0005739	C	mitochondrion	50	1276	1.30E-10	1.20E-08
GO:0009523	C	photosystem II	11	45	2.40E-10	2.20E-08

Table 2 continued.

GO:0009522	C	photosystem I	9	22	2.40E-10	2.20E-08
GO:0010287	C	plastoglobule	13	81	5.50E-10	5.10E-08
GO:0009532	C	plastid stroma	23	322	5.90E-10	5.50E-08
GO:0009570	C	chloroplast stroma	20	249	1.20E-09	1.10E-07
GO:0031975	C	envelope	29	595	1.50E-08	1.30E-06
GO:0031967	C	organelle envelope	29	595	1.50E-08	1.30E-06
GO:0009538	C	photosystem I reaction center	6	11	6.90E-08	6.40E-06
GO:0009526	C	plastid envelope	19	331	4.60E-07	4.30E-05
GO:0005730	C	nucleolus	15	209	5.50E-07	5.10E-05
GO:0010319	C	stromule	8	49	1.10E-06	9.80E-05
GO:0005576	C	extracellular region	17	285	1.10E-06	0.0001
GO:0070013	C	intracellular organelle lumen	24	539	1.20E-06	0.00011
GO:0043233	C	organelle lumen	24	539	1.20E-06	0.00011
GO:0031974	C	membrane-enclosed lumen	24	546	1.50E-06	0.00014
GO:0048046	C	apoplast	13	182	3.30E-06	0.0003
GO:0044425	C	membrane part	41	1360	5.00E-06	0.00046
GO:0030095	C	chloroplast photosystem II	5	29	9.40E-05	0.0087
GO:0022627	C	cytosolic small ribosomal subunit	9	130	0.00013	0.012
GO:0015935	C	small ribosomal subunit	10	164	0.00015	0.014
GO:0043234	C	protein complex	38	1443	0.00018	0.016
GO:0031981	C	nuclear lumen	15	374	0.00035	0.032
GO:0009941	C	chloroplast envelope	12	265	0.00048	0.044
GO:0019866	C	organelle inner membrane	11	230	0.00053	0.049

3.4.9 Infra-Red Screening of Arabidopsis Knock-Out Mutants for Differential Stomatal Response to Sl1344

As indicated by microarray analysis, several genes had significant differential expression when infected with SL1344. From all these genes, a few genes were checked for involvement in stomatal response to SL1344 by a new screening technique. Arabidopsis SALK lines of confirmed homozygous knock-out mutants (from ABRC), available in the lab were used for the experiment. To screen for mutants defective in SL1344-related stomatal re-opening, a thermal imaging based method was employed (Mustilli et al. 2002). This method uses infra-red imaging to measure leaf surface temperature. Transpiration causes cooling of the leaf surface, which can be detected by this method. Transpiration occurs when stomata are open and hence cooler leaf surface temperatures can be correlated with open stomata. This method is non-destructive and can be used in conjunction with stomatal assays. To find proteins important in the stomatal response towards SL1344 infection, homozygous knock-out mutants were screened for changes in surface temperature, correlating with guard cell movement.

Eighteen homozygous mutant lines were infected with SL1344 and plants were imaged at 2 hpi and 4 hpi. Out of the 18 lines, 13 showed leaf temperatures different from Col-0 and hence are discussed here. Out of the 13, 7 showed significant temperature difference from Col-0 and 6 shows slight difference from Col-0 (Table 3; Fig. 3.16). In the wild-type plant Col-0, SL1344 causes stomatal closure at 2 hpi and re-opening at 4 hpi, which is seen as hot leaves at 2 hpi and cold leaves at 4 hpi. The homozygous mutant lines were compared with this Col-0 response to find gene products important for stomatal response towards SL1344. Colder leaf temperatures than Col-0 at 2 hpi indicates deficiency in stomatal closure in response to bacterial treatment and hotter temperatures at 2 hpi indicates strong stomatal immunity. The most interesting result was

to compare stomatal response at 4 hpi. Hotter leaf temperatures at 4 hpi than Col-0 indicate that stomatal re-opening in the presence of SL1344 is compromised. This is observed in plants with AT2G46240, AT2G22340, AT2G29500, or AT1G72600 mutated. These gene products must be important for SL1344 induced stomatal re-opening. The annotation of the genes is given in Table 2. Screening of other genes of interest by this method is currently ongoing.

Table 3. Qualitative comparison of leaf surface temperatures of mutant lines to Col-0 at 2 hpi and 4 hpi. Colder temperatures indicate more open stomata than Col-0 and hotter temperature indicate more number of closed stomata as compared to Col-0. Annotation of each gene (TAIR) that is mutated in the SALK line used is also shown.

SALK number	Mutated Gene	Annotation	Significant/slight change
Colder than Col-0 at 2hpi			
SALK_022866C	AT1G09930	OLIGOPEPTIDE TRANSPORTER 2	Significant
SALK_013280C	AT3G09440	Heat shock protein 70 (Hsp 70) family protein	Significant
SALK_071671C	AT4G34400	AP2/B3-like transcriptional factor family protein	Significant
SALK_072866C	AT1G54050	HSP20-like chaperones superfamily protein	Slight
SALK_010259C	AT1G61065	Protein of unknown function	Slight
SALK_043928C	AT3G46230	ARABIDOPSIS THALIANA HEAT SHOCK PROTEIN 17.4	Slight
SALK_085128C	AT4G23210	Encodes a Cysteine-rich receptor-like kinase (CRK13).	Slight
Colder than Col-0 at 4hpi			
SALK_056007C	AT5G26220	ChaC-like family protein	Significant
Hotter than Col-0 at 2hpi			
SALK_015191C	AT2G40180	Encodes PP2C5, a member of the PP2C family phosphatases.	Significant
Hotter than Col-0 at 4 hpi			
SALK_009534C	AT2G46240	A member of Arabidopsis BAG (Bcl-2-associated athanogene) proteins	Significant
SALK_035466C	AT2G22340	unknown protein	Significant
SALK_013435C	AT2G29500	HSP20-like chaperones superfamily protein	Slight
SALK_027897C	AT1G72600	hydroxyproline-rich glycoprotein family protein	Slight

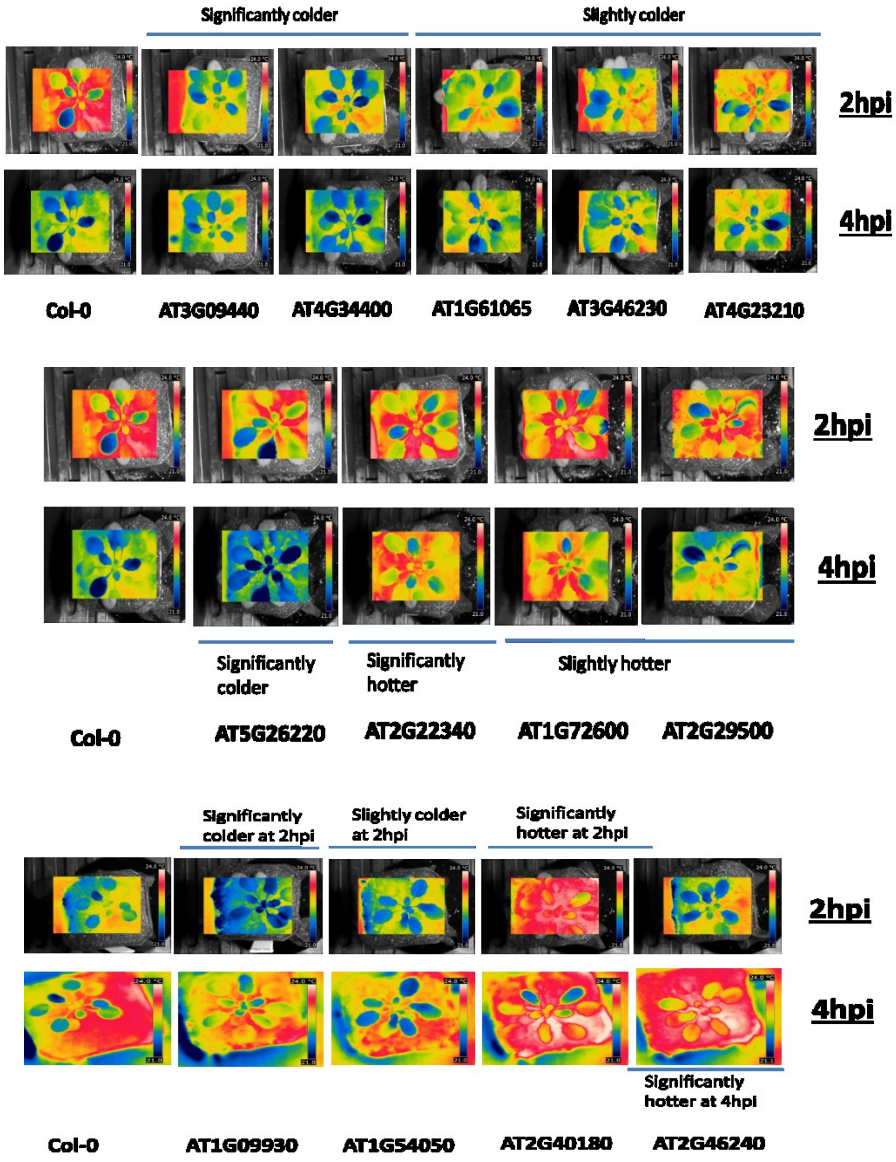

Figure 3.16 Thermal images of Arabidopsis homozygous knock-out mutants obtained 2 h and 4 h after dip-inoculation with 1 x 10^8 CFU/ml^{-1} SL1344.

3.5 Discussion

Plant defense components in Col-0 were analyzed when plants were infected with human pathogens, specifically SL1344. Immunity acting at both pre-internalization and post-internalization of human pathogen in the leaf tissue was studied, by assessing stomatal response and apoplastic defense response.

Overall, the results from this study indicate two possible indications to SL1344 infection in Arabidopsis; either there is weak recognition of the bacteria by the plant and/or SL1344 is able to suppress Arabidopsis defense. These possibilities may or may not be mutually exclusive. The most surprising result was the stomatal response to SL1344. Stomatal immunity in plants relies on recognition of PAMPs, conserved molecules on microbial surfaces, and subsequent stomatal closure to prevent entry of microbes inside the leaf tissue (Melotto et al. 2006). *E.coli* O157:H7, triggers stomatal closure (Melotto et al. 2006; Roy et al. 2013; this study) for at least 8 h (Melotto et al. 2006). Re-opening of stomata in the presence of SL1344 suggests that stomatal immunity is subverted or becomes inactive after 4 hpi. Transient stomatal closure by SL1344 was also observed in iceberg lettuce (Kroupitsi et al. 2009; Roy et al. 2013). Active suppression of stomatal immunity by SL1344 may be unlikely because open stomata were not observed when plants were infected with SL1344 in dark (Roy et al. 2013). However, light was indeed found to be important for SL1344 for chemotaxis towards lettuce stomata (Kroupitski et al. 2009). Hence, influence of light on bacterial behavior or plant physiology cannot be ruled out. In my study, stomatal re-opening was observed only with live bacteria, even when plants were inoculated with very high titers of bacteria. This indicates that some component of live bacterial cells interferes with plant molecular signaling, which may be dependent on light conditions. Preferential internalization through stomata has been a subject of some studies, which indicate that the interaction is not

very generalized and specificities with respect to bacterial strain as well as plant type exist. *Salmonella enterica* serovar Typhimurium SL1344 was shown to internalize arugula and iceberg lettuce through stomata and bacterial cells were located in the sub-stomatal space (Goldberg et al. 2011). However, no internalization of SL1344 was observed into parsley where most cells were found on the leaf surface even though stomata were partially open (Goldberg et al. 2011). Several microscopy studies indicated association of pathogens on or near guard cells. Cells of *S. enterica* serovar Typhimurium MAE110 (Gu et al. 2011), EAEC (Berger et al. 2009a), and *E. coli* O157:H7 (Saldana et al. 2011) were found to be associated with stomata in tomato, arugula leaves, and baby spinach leaves, respectively.

In my study, it was seen that SL1344 colonized Arabidopsis leaf apoplast, but increase in bacterial population over time was not observed. In support of this, *S. enterica* 14028 was also shown to survive but not multiply in Arabidopsis leaf after forced infiltration of bacteria into leaves (Berger et al. 2011). Other studies have also shown that population of enteric bacteria in plants is persistent, but declines with time (Cooley et al. 2003; Islam et al. 2004). A check on population increase indicates the action of some form of plant immunity. Even though it is lesser than O157:H7, SL1344 did induce *PR1* gene, a marker for salicylic acid defense. Salicylic acid-dependent and salicylic acid independent defense responses was shown to be induced by *S. enterica* 14028 in Arabidopsis seedlings (Iniguez et al. 2005), while adult Arabidopsis leaves showed requirement of jasmonate and ethylene signaling for defense against the same strain (Schikora et al. 2008). *Salmonella enterica* infection also induced several components of PTI in tomato and tobacco leaves (Meng et al. 2013) and Arabidopsis seedlings (Garcia et al. 2013). The second factor that can explain lower populations in apoplast is either weak recognition by the plant or bacterial suppression of components of plant immunity. From this study, it is evident that

callose deposition, a marker for apoplastic immunity is compromised. This might be one of the reasons for persistence of bacteria in the leaf apoplast. Interestingly, purified flg22 from *S.* Typhimurium induced callose deposition in Arabidopsis leaves (Garcia et al. 2013). This can suggest either a role of live bacteria to evade plant immunity or lack of recognition of *S.* Typhimurium flagella. Indeed, degradation of flagellin monomers to avoid detection by plant immune system is one of the strategies employed by plant pathogen *Pst* DC3000 (Pel et al. 2014) and opportunistic pathogen *Pseudomonas aeruginisa* (Bardoel et al. 2011). In addition, *PR1* was induced to lesser extent by SL1344 than O157:H7. Hence, bacterial virulence strategies may also play an important role in this fine balance between weak plant immunity and maintenance of population in the apoplast.

Transcriptomic analysis indicated that several genes involved in PTI were commonly regulated when the three datasets of SL1344, O157:H7, and *Pst* DC3000 were compared. A similar transcriptomic analysis with medium-grown Arabidopsis seedlings 2h after inoculation with *S. enterica* serovar Typhimurium 14028, *E. coli* K-12, and *P. syringae* pv. *tomato* DC3000 showed a strong overlap among genes responsive to each bacterial infection suggesting a common mechanism of plant basal response towards bacteria (Schikora et al., 2011). Gene expression analysis of *Medicago truncatula* seedlings root-inoculated with only two bacterial cells per plant indicated that 83 gene probes (30-40% of each data set) were commonly regulated in response to *S. enterica* and *E. coli* O157:H7 (Jayaraman et al., 2014). Specific responses to SL1344 indicated involvement of plant hormones, cell wall modifications, and defense related signaling, all regulated differentially in response to SL1344 infection. Several potential candidate genes can be useful from these results for further analysis to study biological relevance. This study indicates

that the human pathogenic bacterium, SL1344 can modulate specific plant genes beyond a basal defense response; however the mechanisms for largely unknown.

As more studies are being conducted to understand interactions of human pathogen with plants, it is becoming increasingly clear that it is more complicated and specific than previously realized. Differences in cultivars of plants, strains of bacteria, and even age of the plant indicate that the plant response as well as bacterial colonization may not be a generalized phenomenon and that specificity might exist (Berger et al. 2009b; Barak et al. 2011; Barak et al. 2008; Brandl and Amudson, 2008).Research in understanding human pathogens-plant interactions is relatively new and hence we see differences in conclusions from different labs because of differences in methods, strains, plant age. More comparative studies should be done. Since it is well established now that this study of plants with human pathogens is critical as well as complex, consensus and collaborations must be developed between food scientists, microbiologists, and plant pathologists to avoid discrepancies in making general conclusions.

3.6 Conclusion

Infection of *Arabidopsis thaliana* with the human pathogen *Salmonella enterica* serovar Typhimurium SL1344 disrupts components of the plant immune system. Specifically, stomatal immunity, *PR1* defense gene induction, and callose deposition is compromised. The overall plant cellular response to this biotic stress indicates that several components of signal transduction pathways are modulated by SL1344 apoplastic infection. This study indicates that interaction and survival of SL1344 in Arabidopsis leaf tissue is a collective result of weak plant response and/or suppression of immunity by SL1344.

Appendix A

Functional Annotation of Guard Cell Expressed Genes as Discovered by Direct RNA Sequencing

Introduction

Stomatal apertures can open and close in response to several external (light, humidity, CO2) and internal (plant hormones) stimuli. This is brought about by molecular signaling events within the guard cells which allows them to be turgid (closed stoma) or flaccid (open stoma). Until recently, stomata were considered as passive ports on the leaf. But now they have been shown to be immunity gates for the plant, closing in response to pathogen attack on the leaf surface. This is termed as stomatal immunity and is part of the plant innate immune system. Hence, studying signaling mechanisms within guard cells becomes crucial in understanding the effect of abiotic as well as biotic factors on stomatal opening or closing. To study guard cell signaling, it is important to extract and separate out these cells from other parts of the leaf tissue. This study was part of the publication Obulareddy et al. 2013, where a procedure was devised for guard cell protoplasting to avoid induction of biotic stress-associated genes and extensive RNA decay, and to obtain high quality and quantity of RNA useful for studying the effects of biotic stress on the guard cell transcription network through direct RNA sequencing. To determine the efficiency of the protocol discussed, guard cell transcriptomic analysis was performed.

Methods

The analysis was performed on the list of genes obtained after high throughput deep-sequencing of the guard cell transcriptome (RNA-seq). These genes are expressed in the guard cells extracted using the newly devised protocol. Functional annotation of guard cell expressed genes according to plant GO slim categories was retrieved from The Arabidopsis Information Resource database (TAIR10; Arabidopsis.org). Additionally, the AGI (Arabidopsis Genome Initiative) number was used as input for assessing GO enrichment using the Singular Enrichment Analysis (SEA) through AgriGO (http://bioinfo.cau.edu.cn/agriGO/). TAIR10 was used as a

background reference for SEA and statistical significance was detected with Fisher exact test with Benjamini-Hochberg false discovery rate (FDR) correction to calculate the *P* and FDR values. The Illumina RNA-seq data related to this study is available at the NCBI Sequence Read Archive (SRA, Wheeler et al. 2008, http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi) under accession number SRA064368 (mRNA-seq).

Results

Functional categorization of the 18,994 gene transcripts using the GO Slim classification for plants (TAIR10) revealed the GO terms present in our data set that belong to the three broad GOs, Biological Process, Cellular Component, or Molecular Function (Fig. 1). One fourth (25.9%) of the transcripts encode for proteins targeted to the nucleus and chloroplast (Fig. 1A). The most abundant molecular functions include: other binding (14%; excludes nucleic acid and protein binding), transferase activity (13.1%), and hydrolase activity (9.3%) (Fig. 1B). Response to stress and response to biotic and abiotic stimulus accounted for 13% of the biological process annotations (Fig. 1C).

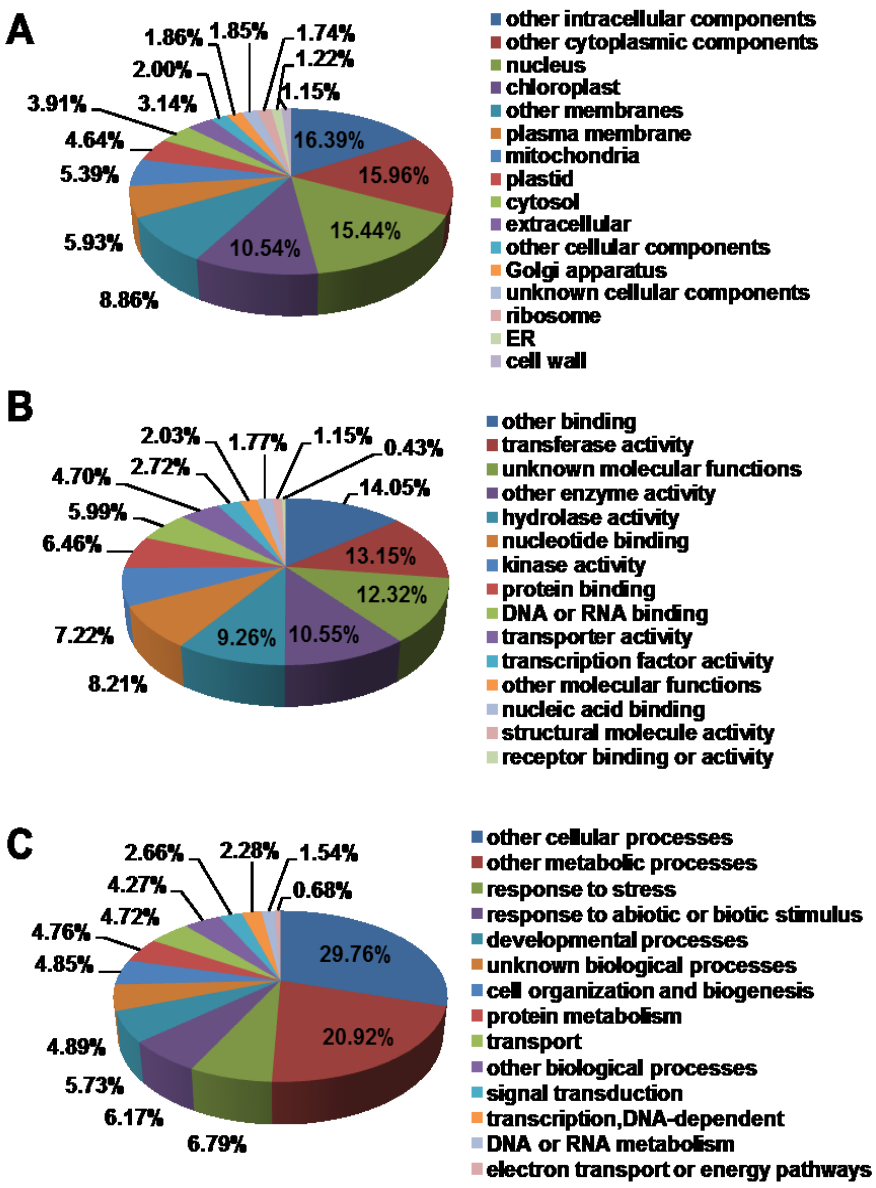

Figure 1. Functional categorization of guard cell expressed genes (18,994) according to the three broad Gene Ontology categories cellular component (A), molecular function (B), and biological process (C) using the GO slim tool available at TAIR.

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