The role of pathogen defense signaling components in ABA signal transduction and isolation of an ABA/C23 signaling mutant

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The Role of Pathogen Defense Signaling Components in ABA Signal Transduction and
Isolation of an ABA/C23 Signaling Mutant

A thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Biology

by

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2009
The Thesis of Tracy Ha is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2009
DEDICATION

This thesis is dedicated to my family, Dr. Tae Houn Kim, Amber Ries, as well as those who supported me throughout my life.
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Chapter One, in part, is being prepared for publication of the material, Kim TH; Ha T; Schroeder JI. The thesis author will be a co-author of this paper.
ABSTRACT OF THE THESIS

Analyses of Pathogen Defense Signaling Mutants in ABA Signal Transduction in Guard Cells and Isolation of an ABA/C23 Signaling Mutant

by

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Professor Julian I. Schroeder, Chair

In a place teeming with pathogens, maintaining a defense mechanism against pathogen attacks is critical for survival especially when other stress factors such as drought can also endanger plant life, a staple of our agriculture. The phytohormone abscisic acid (ABA) regulates major abiotic stress responses, while the hormones
salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) all participate in plant defense against pathogen invasions. Previous research has provided evidence for the antagonistic relationship between ABA signaling and pathogen resistance. To determine the range at which ABA signaling affects plant defense signaling, stomatal responses of pathogenic mutants were examined under treatments of ABA and C23, a synthetic chemical that inhibits ABA signaling and induces expression of defense genes. Results reveal that the convergence points between ABA and defense signaling occur upstream of SA synthesis and signaling. To identify a new mutant that is interconnected between the two pathways, mutant screening and genetic cloning of N277 is performed, a mutant line shown to exhibit insensitivity to ABA and to the C23 pathogen-induced inhibition of ABA signaling. This study aims to understand the mechanism at which ABA signaling interacts with pathogen defense signaling to enhance plant protection from pathogen invasions without decreasing crop yield.
CHAPTER 1:

Characterization of the roles of defense-signaling components in ABA signal transduction
1.1 Introduction

In an environment filled with invasive bacterial pathogens, there are several natural gates of where pathogens have been observed to enter the plant. Among the many passages, the stomata—pores on the epidermis of plants that control gas exchange and water transpiration between the plant interior and environment, may represent the most important entry since stomata dominate in number in the aerial part of the plant (Melotto et al., 2008). A reduction of stomatal apertures was observed when the leaves or epidermal peels were exposed to a suspension of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, a virulent pathogen of tomato and Arabidopsis. These stomata returned to the open state after 3 hours of incubation. (Katagiri et al., 2002; Whalen et al., 1991). Here, observations show that stomata are not completely passive ports of entry for bacteria, suggesting that plants have evolved mechanisms to reduce the penetration of bacteria as an essential part of plant immunity, and bacteria have developed a counter response to alleviate the plant resistance mechanism (Melotto et al., 2008).

*Pst* DC3000 is characterized by two virulence factors: the hrc/hrp gene-encoded type III secretion system (TTSS) that delivers effector proteins (Alfano and Collmer, 2004), and the phytotoxin coronatine (COR) into the host cell (Bent and Mackey, 2007). While the *hrc* mutant can re-open closed stomata, COR-deficient mutants have been proposed, suggesting that coronatine is a factor involved in suppressing stomatal defense (Melotto et al., 2006). Several pathogenic variants of *P. syringae* produce coronatine, which is structurally similar to the phytohormone jasmonic acid (JA) in pathogen signaling (Schulze-Lefert and Robatzek, 2006).
In the line of defense against pathogens, the first step for the cell is to recognize the presence of pathogens, which come in the form of pathogen-associated molecular patterns (PAMPs) (Ryan et al., 2007). Plants perceive PAMPS through membrane-integrated leucine-rich repeat (LRR) receptors (Bent and Mackey, 2007), such as the flagellin receptor FLS2 (Chinchilla et al., 2007).

Although the mechanisms of abiotic and biotic stresses have usually been studied as separate entities, emerging evidence has revealed that there is an antagonistic interaction between biotic and abiotic stress signaling. The phytohormone abscisic acid (ABA) triggers in responses to the abiotic stresses of drought and osmotic stress, as well as growth and developmental processes of seed development, dormancy, and germination (Shinozaki et al., 2003; Fujita et al., 2006). One of the many important roles of ABA is that it regulates the stomata opening and closure (Schroeder et al., 2001). The stomata are tiny pores located on the epidermis of leaves and stems, bounded by a pair of guard cells which regulate their opening and closing to maintain a balance of CO2 intake and transpirational water loss in response to their environment (Hetherington and Woodward, 2003; Israelsson et al., 2006). The presence of light hyperpolarizes the plasma membrane by stimulating H+-ATPases, allowing the influx of K+ through voltage-dependent K+ channels (Schroeder et al., 1987). Water then follows through and increases the turgor pressure that drives the opening of stomata (Roelfsema and Hedrich, 2005). Additionally, the transport of anions including NO3 and Cl into the guard cells and malate produced from starch all contribute to the osmotic pressure to increase the turgidity of guard cells (Kwak et al., 2008). Although the molecular events during the
opening of guard cells may seem simple, the signaling cascades during ABA-induced stomatal closure is extensive and many processes have been studied.

While increasing turgor pressure opens the stomata, reducing the turgor pressure closes the stomata (Kwak et al., 2008; Sirichandra et al., 2009). Turgor pressure reduction accompanied by effluxes of K\(^+\) and anions from guard cells, sucrose removal, and the conversion of malate to osmotically inactive starch (MacRobbie, 1998; Schroeder et al., 2001). The depolarization of the plasma membrane from the activation of anion channels and inhibition of H\(^+\)-ATPases causes the efflux of K\(^+\) and anion release to decrease the turgor pressure of guard cells (Sirichandra et al., 2009; Keller et al., 1989; Schroeder and Hagiwaga, 1989; Shimizaki et al., 2007). During times of drought, plants synthesize ABA as an endogenous signal to trigger an increase in cytosolic Ca\(^{2+}\) via plasma membrane Ca\(^{2+}\) ion channels and Ca\(^{2+}\) release from internal reservoirs, leading to the activation of anion channels and membrane depolarization (McAinsh et al., 1990; Schroeder et al., 2001). The membrane depolarization down-regulates inward-rectifying K\(^+\) channels (Schroeder et al., 1987; Pilot et al., 2001) and activates outward-rectifying K\(^+\) channels (Kwak et al., 2008; Schroeder et al., 1987; Hosy et al., 2003). Through the efflux of anions and K\(^+\) from guard cells in the presence of ABA, the guard cells lose turgor and close the stomata (Schroeder and Hagiwara, 1989).

As for the phytohormones of pathogen signaling—salicylic acid (SA), jasmonic acid (JA), and ethylene (ET)—have functions in biotic stress signaling upon pathogen attack (Fujita et al., 2006). In tomatoes ABA-deficient tomato mutant _sitiens_ exhibited increased resistance to pathogens, but the susceptibility of _sitiens_ was restored after the exogenous application of ABA (Audenaert et al., 2002; Thaler and Bostock, 2004). The
SA-mediated response was stronger in the *sitiens* mutant than in wildtype, implying that high ABA concentrations inhibit the SA-mediated defense response in tomato. In *Arabidopsis* increased ABA concentrations from ABA treatment or simulated drought stress resulted in enhanced susceptibility to pathogenic bacteria (Mohr and Cahill, 2003). Further studies with exogenous application of ABA, inhibition of ABA biosynthesis, or with ABA-deficient mutants have shown a correlation between elevated ABA levels and increased susceptibility to pathogen infection (Mauch-Mani and Mauch, 2005).

Research has also shown that ABA interacts antagonistically to ethylene and jasmonic acid (Beaudoin et al., 2004; Anderson et al., 2004). Analyzing enhanced response to ABA3 (*era3*) alleles show that *ERA3* is allelic to *ETHYLENE INSENSITIVE2* (*EIN2*), representing a crosstalk between ABA and ethylene signaling pathways (Ghassemian et al., 2000). Moreover, *jasmonic acid resistance1* (*jar1*) and *jasmonic acid insensitive4* (*jin4*) mutants, hypersensitive to ABA-induced inhibition of germination, have antagonistic effects between ABA and jasmonic acid (Lorenzo and Solano, 2005; Anderson et al., 2004). While the exogenous application of ABA leads to downregulation of jasmonic acid- or ethylene-dependent defense gene expression in wildtype plants, ABA-deficient mutants without any treatments had higher expression levels of the defense genes (Anderson et al., 2004). However, the inability for the exogenous application of methyl-jasmonic acid and ethylene to restore the defense gene expression, after suppression from the exogenous application of ABA, suggests that the ABA-mediated abiotic stress response is more dominant (Anderson et al., 2004).
The versatility of ABA is not limited to an antagonistic interaction with pathogen defense signaling, they also share a synergistic relationship. The *Arabidopsis* protein *BOTRYTIS SUSCEPTIBLE1* (BOS1) shares a high sequence similarity with AtMYB2, another transcriptional activator in ABA signaling (Mengiste et al., 2003). Disrupting BOS1 increased susceptibility to necrotrophic pathogens and weakened tolerance towards drought, salinity, and oxidative stress (Mengiste et al., 2003). The bipolar interaction between ABA and pathogen defense signaling implies the presence of a sophisticated network to cope with abiotic and biotic stresses.

Previous research has recognized that parts of fungal and bacterial cell walls can elicit an immunity response in plants (Ebel and Cosio, 1994; Boller, 1995). These components have included β-glucan elicitors, chitin fragments, lipopolysaccharides, glycopeptides and proteins, which all serve as PAMPs to interact with receptors located on either the plant’s cell surface or in the cytoplasm to stimulate rapid defense responses against pathogens (Ryan et al., 2007). When the fragments of protein flagellin, a component of bacteria mobility, is released from the bacteria, its N-terminal region can elicit an alkalization of the medium of cells, a sign of an innate immune response (Felix et al., 1999). From *Pseudomonas syringae* pv. *Tabaci*, the N-terminus of flagellin was found to contain a conserved 22-amino-acid peptide, flg22 (Felix et al., 1990). When flg22 is administered in Arabidopsis seedlings, it caused callose formation, inhibition of growth, and the expression of defense genes that are activated through ethylene and salicylic acid signaling pathways (Gómez-Gómez et al., 1999; Zipfel et al., 2004). The extracellular recognition of PAMPs induce PAMP-triggered immunity (PTI), which lead
to the mobilization of MAPK signaling, transcriptional reprogramming, production of reactive oxygen species, and callose deposition (Nurnberger and Kemmerling, 2006).

In addition to the PAMP-triggered immunity, the major mechanism that plants use to provide defense against pathogens is through gene-for-gene resistance, which is a pathogen recognition system (Dodds and Schwechheimer, 2002). This uses intracellular plant resistance (R) proteins to detect pathogen effectors transported into host cells during infection, leading to an effector-triggered immunity (ETI) (Liu et al., 2009). The R genes signaling products lead to the recognition of pathogen avirulence (Avr) proteins (Martin, 1999). The interaction occurs either within or on the surface of plants, leading to a number of responses including localized plant cell necrosis, or hypersensitive response, an oxidative burst that produces reactive oxygen intermediates, an accumulation of nitric oxide and salicylic acid, and the transcriptional activation of resistance-related genes (Kjemtrup et al., 2000; McDowell and Dangl, 2000). The majority of R genes can be categorized into a family of encoding proteins with a nucleotide-binding site (NBS) and C-terminal LRR domains (Meyers et al., 2003). Most Arabidopsis NBS-LRR genes encode a protein with an N-terminal domain that have sequences similar to the cytosolic domain of animal innate immune transmembrane receptors, Toll and Interleukin-1 Receptor (TIR) (Martin et al., 2003). Another category of NBS-LRR R proteins have an N-terminal coiled-coil (CC) domain (Martin et al., 2003; Meyers et al., 2003; Nimchuk et al., 2003).

Although the distinction between TIR-NBS-LRR (TNL) and CC-NBS-LRR (CNL) pathways is not absolute, TNL proteins require functional ENHANCED DISEASE
SUSCEPTIBILITY1 (EDS1) and PHYTOALEXIN DEFICIENT4 (PAD4) genes, while CNL proteins require NONRACE SPECIFIC DISEASE RESISTANCE1 (NDR1) to activate further downstream pathogen signaling pathways (Aarts et al., 1998). Among the CNL genes, the first Arabidopsis R genes to be cloned were RPS2, RPM1, and RPS5 (Bent and Mackey, 2007; Grant et al., 1995; Warren et al., 1998). However large the TNL class of R proteins is, only RPS4, known to recognize a Pseudomonas syringae effector, has been characterized in detail (Hinsch and Staskawicz, 1996).

The Arabidopsis EDS1 and PAD4 genes, which encode for lipase-like proteins, participate in the same signaling pathway by forming a protein complex but also have different roles (Aarts et al., 1998; Feys et al., 2001). Analyzing RPS4-specific responses reveal that EDS1 operates upstream of SA-dependent defenses (Falk et al., 1999). Mutations in EDS1 abolish RPS4-mediated resistance to Pseudomonas syringae expressing avrRps4 (Aarts et al., 1998) and the eds1 mutant plants are hypersusceptible to P.syringae and P.parasitica (Parker et al., 1996; Aarts et al., 1998). Furthermore, the dimerization of EDS1 has also been present during a resistance response (Feys et al., 2001). PAD4 was the first identified component for enhanced disease susceptibility to a virulent isolate of P.syringae pv. Maculicola, PAD4 was required for resistance conferred by RPP2 and RPP4 to P.parasitica in Col-0 cotyledons (Glazebrook et al., 1997). EDS1 is essential for developing a hypersensitive response and for accumulating pathogen-induced PAD4 mRNA; however, both the association between EDS1 and PAD4 is required to accumulate SA (Feys et al., 2001). The application of SA upregulates the abundance of EDS1 and PAD4 mRNAs, implying the presence of a
positive feedback loop in the expression of these genes (Falk et al., 1999; Jirage et al., 1999).

While EDS1 and PAD4 function together as a complex under the TIR-NBS-LRR class of R genes, RAR1 and SGT1b are associated with each other under the CC-NBS-LRR class of R genes (Martin et al., 2003). By having an enhanced cell wall defense response against flg22, rar1 mutants indicate that RAR1 negatively regulates basal defense and plays a role in both PTI and ETI (Shang et al., 2006). The barley RAR1 gene is needed for powdery mildew resistance at the Mla resistance locus and other R loci (Dodds and Schwechheimer, 2002). *The P. syringae* effector protein AvrB suppresses PAMP-triggered immunity through RAR1, a cochaperone of HSP90 necessary for effector-triggered immunity (Shang et al., 2006). Both RAR1 and HSP90 bind to SGT1, a conserved eukaryotic protein with functions in many biological processes through interactions with protein complexes (Takashi et al., 2003; Shirasu and Schulze Lefert, 2003). The RAR/SGT1b complex plays a role in protein degradation when RAR1 was found to coimmunoprecipitate with the COP9 signalosome, a complex that functions in protein degradation (Dodds and Scwechhemier, 2002; Azevedo et al., 2006). In plants SGT1 positively regulates R protein-mediated resistance via R protein accumulation (Azevedo et al., 2006). *Arabidopsis* has two SGT1 isoforms, AtSGT1a and AtSGT1b; both function in resistance, but AtSGT1b levels are higher in steady-state conditions (Azevedo et al., 2006; Austin et al., 2002). The range of R genes that require RAR1 or SGT1 do not overlap with those that use EDS1/PAD4 (Dodds and Schwechheimer, 2002).
Unlike the gene-for-gene resistance that requires a specific interaction between the $R$ gene and the corresponding $Avr$ gene, the systemic acquired resistance (SAR), provides a non-specific and long-lasting induced resistance to a wide range of pathogens (Ryals et al., 1994). Characteristics of SAR are the accumulation of pathogenosis-related (PR) proteins and SA, a key signaling molecule in SAR (Ryals et al., 1996). The transgenic plant-expressing salicylate hydroxylase ($nahG$) blocks SAR and prevents PR gene expression by converting SA into catechol, rendering it inactive (Delaney, 1997; Mauch-Mani and Metraux, 1998). The defense mechanism is divided into two signaling pathways downstream of SA: one elicits resistance to bacteria and fungi by $NPR1$ and PR gene expression and another induces resistance to viral infection by alternative oxidase (AOX), a plant mitochondrial enzyme (Murphy et al., 1999). The SAR response was also impaired in $eds16/sid2$ mutants deficient in pathogen-induced SA accumulation (Nawrath and Métraux, 1999). A simplified model of the mechanism of pathogen signaling can be viewed in Figure 1.
Figure 1: A simplified model of the defense signaling mechanism.
Although the involvement of pathogen defense signaling and its branches in abscisic acid signaling have been progressively shown in recent years, the molecular mechanisms and the overlap between the signaling pathways remain unclear. This chapter aims to characterize the roles of defense-signaling components in ABA signal transduction. An instrumental chemical used throughout this research was Compound 23 (C23), a synthetic novel chemical that inhibits ABA signaling (Kim and Schroeder, unpublished). Furthermore, microarray-based transcriptome analyses revealed that C23 treatment can increase transcripts levels of genes involved in the defense signaling pathway; hence, besides other unknown functional mechanism of C23, the application of C23 can result in mimicking the effect of pathogen invasion (Kim and Schroeder, unpublished). Inspired from previous studies that provided evidence of the interactions between ABA and defense signaling, the mutants pad4-1, eds1, rar1-21, sgt1b, npr1-1, eds16-1, and nahG, that are defective in defense signaling, were investigated. As functional components of defense signaling, these mutants represent the different branches in the network of defense signaling against pathogens; therefore, specific areas in the signaling network can be focused and targeted for analyses. To investigate the hypothesis that pathogen signaling is involved in ABA signal transduction and to further understand which components and signaling branches of defense signaling can affect ABA signaling in guard cells, the stomatal responses of pad4-1, eds1, rar1-21, sgt1b, npr1-1, eds16-1, and nahG mutants are analyzed under treatments with ABA and C23. By expanding the beginning knowledge between the dynamics of ABA and defense signaling, the information can be hopefully applied to improving crop health and yield by
enhancing plant defenses against pathogens with minimal effects to resistance mechanism against abiotic stress.

1.2 Materials & Methods

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) Col-0 wildtype and mutant seeds were sown in plastic pots filled with ready-to-use soil (Professional Blend). The pots were kept at 4ºC for 3 to 5 days in the dark for stratification. They were then placed in a Conviron ® plant growth chamber and allowed to grow. The following growth conditions were as follows: 22ºC, 75% humidity with a 16-hour light/8-hour dark cycle at approximately 80 μmol m⁻² s⁻¹.

Stomatal Closing Aperture Measurements

To observe stomatal movement, the abaxial epidermis needed to be extracted and isolated on a cover slip. A leaf from 4-5 weeks old plant was mounted onto a cover slip using Hollister Medical Adhesive glue (CA# 7730, Hollister, Inc.). The mesophyll tissue was scraped off to leave behind a 2-cell layer thick of epidermis on the cover slip. Cover-slips mounted with the leaf epidermis were incubated in buffer (10 mM KCl, 7.5 mM iminodiacetic acid, 10 mM MES and pH 6.2 adjusted with KOH) for 1.5 hours under light, and replaced with opening buffer containing 0 μM ABA, 50 μM ABA, 30 μM C23, or 50 μM ABA + 30 μM C23 for 1.5 hours. Samples containing any C23 were pre-treated with 30 μM C23 30 min before the addition of treatments. Cover-slips were then
transferred to a microscope slide for viewing and measuring under the light microscope. Experiments were double-blinded in which the identities of the genotype and treatment were unknown at the time of experiment.

**Stomatal Opening Aperture Measurements**

Pots with plants were first kept in the dark overnight by being wrapped in aluminum foil. To observe stomatal movement, the abaxial epidermis was isolated onto a cover slip as previously mentioned. Epidermal leaf samples mounted on cover-slips were submerged in buffer (10 mM KCl, 7.5 mM iminodiacetic acid, 10 mM MES and pH 6.2 adjusted with KOH) and wrapped with aluminum foil to prevent light penetration for 2 hours at 20°C. After 2 hours of incubation in the dark, the samples were exposed to light with a fluence rate of 160 µmol m⁻² s⁻¹ and replaced with buffer containing 0 µM ABA, 20 µM ABA, 30 µM C23, or 20 µM ABA + 30 µM C23. Measurements of the stomatal apertures under the microscope followed after 2 hours of incubation under the light with treatments. Experiments were performed double-blinded in which the identities of the genotype and treatments were unknown at the time of experiment.

### 1.3 Results

*The pad4 and eds1 mutants display insensitivity to C23 inhibition of ABA signaling in stomatal closing experiments.*

To determine whether inhibition of ABA signal transduction by C23 is affected in the pathogenic mutant pair of *eds1* and *pad4*, ABA-induced stomatal closing experiments
were performed with treatments of 0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23 (Figure 2). Upon examination of eds1 and pad4 with Col-0 wildtype as a control for ABA responses, stomatal responses to ABA were not as severely impaired. The stomatal apertures of eds1 and pad4 were not significantly different from that of wildtype with ABA treatments (Figure 2). When C23 was added together with ABA, ABA signaling was inhibited in Col-0 wildtype and stomatal apertures were more opened than without C23 treatment. However, in eds1 and pad4 mutants, ABA signaling was still active and induced stomatal closure under the combined treatment ABA and C23, showing insensitivity to C23 inhibition of ABA signaling. This result indicated that the major pathogen defense signaling regulator EDS1 and PAD4 are required for C23 inhibition of ABA signal transduction.
Figure 2: The *eds1* and *pad4* mutants exhibit insensitivity to C23 inhibition of ABA signaling. a) ABA-induced stomatal closing aperture measurements of wildtype (Col-0) and *eds1* in treatments (0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error. b) ABA-induced stomatal closing aperture measurements of wildtype (Col-0) and *pad4* in treatments (0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error. Experiments were double-blinded.
The rar1-21 and sgt1b mutants pair display insensitivity to C23-inhibition of ABA signaling in stomatal closing experiments.

To determine if the mutant pair rar1-21 and sgt1b is susceptible to C23 inhibition of ABA signaling, stomatal closing experiments were performed with 0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23 (Figure 3). Stomatal responses to ABA were still intact in rar1-21 and sgt1b. However, rar1-21 and sgt1b resulted closed stomatal apertures in the presence of both ABA and C23 (Figure 3). These results reveal that rar1-21 and sgt1b are insensitive to C23 inhibition of ABA signaling, indicating RAR1 and SGT1B have roles in C23-inhibition of ABA signaling.
Figure 3: The *rar1-21* and *sgt1b* mutants exhibit insensitivity to C23 inhibition of ABA signaling. a) ABA-induced stomatal closing aperture measurements of wildtype (Col-0) and *rar1-21* in treatments (0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error. b) ABA-induced stomatal closing aperture measurements of wildtype (Col-0) and *sgt1b* in treatments (0µM ABA, 50µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error. Experiments were double-blinded.
The *npr1-1* mutant has a normal response to C23-inhibition of ABA signaling in stomatal closing experiments.

To determine whether the C23 inhibition of ABA signaling extends into the SA-dependent defense signaling pathway, stomatal responses of *npr1-1* were analyzed. Stomatal closing experiments with treatments of 0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23 were performed on *npr1-1* (Figure 4). Results reveal that *npr1-1* behaved similar to Col-0 wildtype when stomatal apertures of both *npr1-1* and Col-0 remained open in the presence ABA and C23. Therefore, ABA signaling is not disrupted by the inhibition of C23 in *npr1-1*, suggesting that NPR1 is not required for C23 inhibition of ABA signaling and C23-inhibition occurs upstream of *NPR1*. 
Figure 4: The *npr1-1* mutant has a wildtype response to C23 inhibition of ABA signaling. ABA-induced stomatal closing aperture measurements of wildtype (Col-0) and *npr1-1* in treatments (0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23). N=5 experiments, 30 stomata per treatment. Error bars denote standard error. Experiments were double-blinded.
The *eds16-1* mutant contains a normal response C23-inhibition of ABA signaling in stomatal closing experiments.

To determine whether C23 inhibition of ABA signaling is affected in *eds16-1*, stomatal closing experiments with treatments of 0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23 were performed on *eds16-1* (Figure 5). Results show that *eds16-1* behaved similarly to Col-0 wildtype when stomatal apertures of both *eds16-1* and Col-0 remained open in the presence ABA and C23. Thus, inhibition by C23 in ABA signaling is not impaired in *eds16-1*, indicating that *EDS16* is not required for C23 signaling and ABA signaling inhibition by C23 occurs upstream of *EDS16*. 
Figure 5: The *eds16-1* mutant is defective in C23-inhibition of ABA signaling. ABA-induced stomatal closing aperture measurements of wildtype (Col-0) and *eds16-1* in treatments (0 μM ABA, 50 μM ABA, 30 μM C23, 50 μM ABA + 30μM C23). N=5 experiments, 30 stomata per treatment. Error bars denote standard error. Experiments were double-blinded.
The nahG transgenic line has a normal response C23-inhibition of ABA signaling in stomatal closing experiments.

Since bacterial enzyme nahG degrades salicylic acid, the nahG transgenic line contains less SA level compared to wildtype. To determine if the C23 inhibition of ABA signaling is impaired in the nahG transgenic line, stomatal closing experiments with treatments of 0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23 were performed on a transgenic line expressing the bacterial nahG enzyme (Figure 6). Like npr1-1 and eds16-1, inhibition of ABA signaling by C23 was not impaired in the nahG transgenic line because stomatal apertures of the nahG transgenic line remained open in the presence ABA and C23. This suggests that the presence of salicylic acid or induction of salicylic acid is not required for C23 inhibition of ABA signaling.
Figure 6: A transgenic line expressing *nahG* exhibits normal sensitivity to C23-inhibition of ABA signaling. ABA-induced stomatal closing aperture measurements of wildtype (Col-0) and a *nahG* line in treatments (0 µM ABA, 50 µM ABA, 30 µM C23, 50 µM ABA + 30 µM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error. Experiments were double-blinded.
C23 can interfere with the ABA-inhibition of stomatal opening responses and EDS1 and PAD4 are required for this C23 inhibition activity.

In addition to the stomatal closing experiments, stomatal opening experiments were performed, first, to examine whether C23 can intervene the ABA-inhibition of stomatal opening in wildtype. Consistent with the stomatal closing experiment, C23 inhibited the ABA activity in wildtype during stomatal opening (Figure 7). To ensure that stomata were completely closed before ABA treatment, stomatal apertures were measured before inducing stomatal opening. Therefore, the sole function of ABA in these light inducing stomatal opening experiments was to inhibit stomatal opening without further inducing stomatal closure. Then, in order to test whether the C23 inhibitory effect is present during ABA-inhibition of stomatal opening, the mutant pair eds1 and pad4 were analyzed with treatments of 0 µM ABA, 20 µM ABA, 30 µM C23, 20 µM ABA + 30 µM C23 (Figure 7). The eds1 and pad4 mutants were still sensitive to ABA-inhibited stomatal opening in the presence C23-inhibition of ABA signal transduction. Hence, C23 inhibits ABA signaling in ABA-induced stomatal closures and ABA-inhibited stomatal opening, and the major defense signaling regulators, EDS1 and PAD4, are required to mediate the inhibition of ABA signaling by C23.
Figure 7: Stomatal opening experiments of *eds1* and *pad4* confirm the insensitivity to C23-inhibition of ABA signaling. a) ABA-inhibited stomatal opening aperture measurements of wildtype (Col-0) and *eds1* in treatments (0 μM ABA, 20 μM ABA, 30 μM C23, 20 μM ABA + 30 μM C23). N=3 experiments, 30 stomata per treatment. b) ABA-inhibited stomatal opening aperture measurements of wildtype (Col-0) and *pad4* in treatments (0 μM ABA, 20 μM ABA, 30 μM C23, 20 μM ABA + 30 μM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error. Experiments were double-blinded.
Stomatal opening experiments confirm the presence of insensitivity to C23 inhibition of ABA signaling in rar1-21 and sgt1b mutants.

Likewise, stomatal opening experiments were performed on the mutants rar1-21 and sgt1b with treatments of 0 μM ABA, 20 μM ABA, 30 μM C23, 20 μM ABA + 30 μM C23 to determine if C23’s inhibitory effect occurs during ABA-inhibition of stomatal opening (Figure 8). Comparing the measurements of stomatal apertures before exposure to light to the stomatal apertures with ABA treatment indicated that all stomata were closed and ABA did not induce further stomatal closure. These results revealed that ABA-inhibited stomatal opening was still present in rar1-21 and sgt1b, in spite of the C23 inhibitory effect on ABA signaling. Therefore, RAR1 and SGT1B are required for the inhibition of ABA signal transduction by C23.
Figure 8: Stomatal opening experiments of *eds1* and *pad4* confirm the insensitivity to C23 inhibition of ABA signaling.  

a) ABA-inhibited stomatal opening aperture measurements of wildtype (Col-0) and *rar1-21* in treatments (0 µM ABA, 20 µM ABA, 30 µM C23, 20 µM ABA + 30 µM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error.  

b) ABA-inhibited stomatal opening aperture measurements of wildtype (Col-0) and *sgt1b* in treatments (0 µM ABA, 20 µM ABA, 30 µM C23, 20 µM ABA + 30 µM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error. Experiments were double-blinded.
Stomatal opening experiments of npr1-1 confirm npr1-1 does not abrogate C23 inhibition of ABA signaling.

To determine if C23’s inhibitory effect occurs during ABA-inhibition of stomatal opening in npr1-1, stomatal opening experiments were performed with treatments of 0 µM ABA, 20 µM ABA, 30 µM C23, 20 µM ABA + 30 µM C23 (Figure 9). Treatments of ABA and C23 in npr1-1 revealed that ABA-inhibited stomatal opening was disrupted by the inhibition of C23. Therefore, npr1-1 is sensitive to C23 inhibition of ABA signaling, indicating that NPR1 is not a required component for C23 inhibition of ABA signal transduction.
Figure 9: Stomatal opening analyses of npr1-1 confirm the sensitivity to C23-inhibition of ABA signaling. ABA-inhibited stomatal opening aperture measurements of wildtype (Col-0) and npr1-1 in treatments (0 µM ABA, 20 µM ABA, 30 µM C23, 20 µM ABA + 30 µM C23). N=3 experiments, 30 stomata per treatment. Error bars denote standard error. Experiments were double-blinded.
1.4 Discussion

Having a defense mechanism against pathogenic invasions is a critical component for survival and fitness in plants in addition to dealing with abiotic stresses such as drought, low temperatures, and osmotic stress. Research on defense signaling and ABA signal transduction have usually been studied separately as independent subjects, however, recent studies have revealed convergence points among the molecular mechanisms of biotic and abiotic stress signaling. The focus of this research is to study the crosstalk between plant defense and ABA signaling by observing the stomatal responses of pathogenic mutants upon treatments of ABA and C23, a synthetic compound that induces transcription levels of defense signaling.

An interaction between ABA and C23-induced defense signaling involves EDS1, PAD4, RAR1, and SGT1B

The Arabidopsis mutants eds1, pad4, rar1, and sgt1b have previously been shown to have major regulatory roles in plant defense signaling; however, their association with ABA-induced stomatal closure or any ABA signal transduction is unclear (Aarts et al., 1998; Feys et al., 2001; Shang et al., 2006). Previous studies have revealed a correlation between elevated ABA levels and heightened susceptibility to pathogen infection, stating that ABA interacts antagonistically to pathogen defense signaling (Mauch-Mani and Mauch, 2005; Anderson et al., 2004; Fan et al., 2009). Since C23 induces pathogen defense signaling at the transcription level (Kim and Schroeder, unpublished), C23 treatment mimics the effects of a pathogen attack and provide a tool to investigate an interaction between ABA and pathogen signaling.
In wildtype, ABA signaling is inhibited by the application of C23; consequently, ABA-induced stomatal closures are reduced in the presence of C23. In the case of the eds1, pad4, rar1, and sgt1b mutants, stomatal apertures of those mutants were closed in the presence of both ABA and C23 (Figure 2 and 3), indicating that ABA signaling is not inhibited by the presence of C23. Considering C23 does not induce defense signaling in eds1, pad4, rar1, and sgt1b (Kim and Schroeder, unpublished), this observation further suggests that C23-induction of defense signaling contributes to inhibit ABA signaling, supporting previous studies in which ABA signal transduction and plant defense signaling shared an antagonistic relationship.

To confirm the unprecedented results from stomatal closure assays, ABA-inhibited stomatal opening experiments were performed on the eds1, pad4, rar1, and sgt1b mutants in the presence of ABA and C23, another alternative ABA response to illustrate the relationship between ABA and defense signaling. ABA not only induces stomatal closure, it also inhibits stomatal opening as well (Kwak et al., 2008). After closing stomata by overnight dark treatment, the stomatal apertures of wildtype opened after exposure to light under treatments of both ABA and C23, indicating that the induction of defense signaling via the application of C23 inhibited ABA signal transduction, which prevented ABA from inhibiting stomatal opening. In the eds1, pad4, rar1, and sgt1b mutants, opening of stomatal apertures were reduced under treatments of ABA and C23, indicating that ABA signaling prevailed even with the application of C23 (Figure 7 and 8). Hence, these stomatal opening experiments confirm previous observations that defense signaling interacts antagonistically to ABA signal transduction.
Furthermore, the crosstalk between ABA and defense signaling must involve *EDS1*, *PAD4*, *RAR1*, and *SGT1B*.

**Crosstalk between ABA and C23-induced defense signaling occurs upstream of salicylic acid signaling**

In order to examine whether SA is responsible for this antagonistic interaction with ABA signaling, mutants with specific defects in SA biosynthesis and signaling were tested for C23 inhibition of ABA signaling. The *Arabidopsis npr1-1, eds16-1* mutants and *nahG* expressing transgenic lines have previously been studied to be involved in SA-dependent defense signaling (Murphy et al., 1999; Delaney, 1997; Métraux, 1999). Likewise, stomatal closing experiments were performed on these mutants. However, stomatal closing experiments reveal that the *npr1-1, eds16-1*, and *nahG* mutants behaved like wildtype in which stomatal closures were reduced in response to ABA and C23, suggesting that the ABA signaling was successfully inhibited by C23 (Figure 4-6). In addition, stomatal opening experiments on *npr1-1* revealed that *npr1-1* behaved like wildtype in which ABA signaling was inhibited by C23, allowing stomata to be more opened under exposure to light in the presence of ABA and C23 (Figure 9). Therefore, the inhibition of ABA signaling by C23 was not impaired in the *npr1-1, eds16-1*, and *nahG* mutants, suggesting that the crosstalk between ABA and defense signaling occurs upstream of SA synthesis and signaling. As future research, live bacterial strains of *Pseudomonas syringae* tomato DC3000 can be employed in substitution of C23 to ensure induction of pathogen defense signaling in ABA-induced stomatal closing experiments (Katagiri et al., 2002; Whalen et al., 1991).
Implications of findings

This thesis study provides evidence that the induction of pathogen defense signaling by C23 inhibits ABA signaling, and the crosstalk between the two signaling pathways involves *EDS1*, *PAD4*, *RAR1*, and *SGT1B* in a salicylic acid-independent manner. Upon induction of pathogen defense signaling by C23, ABA signal transduction was inhibited, implying that the interaction between pathogen resistance and ABA signaling is antagonistic. This antagonistic interaction suggests that in the presence of both abiotic and biotic stresses, specifically pathogen invasion and drought-mediated stress, plants will suppress their ABA-mediated regulation and divert their focus into building a strong defense against pathogen attack to increase survival. Although inhibiting ABA signal transduction may seem counter-productive, this may be necessary for plants to recruit energy to mount a defense response, when the pathogen invasion is more immediate and detrimental than the consequences of water deficiency during a drought stress. After the plant has successfully resisted a pathogen attack and survived, the plant—although weakened by the suppression of ABA-regulated function—can hopefully recover by reinstating ABA signal transduction. The antagonistic interaction between pathogen defense and ABA signaling suggests the need of a balance between the two in which plant defense is strong enough to protect the plant from pathogen attacks, with minimal impairment to plant growth and development upon inhibition of ABA signal transduction. Understanding the molecular mechanism by which ABA signaling interacts with defense signaling will be helpful towards establishing this balance to increase the plant immunity from pathogen invasion without disrupting crop yield.
To discover more components that are required for the C23-induction of ABA-signaling inhibition, or genes that have roles simultaneously in ABA and defense signaling, mutants exhibiting insensitivity to C23-inhibition of ABA signaling were screened and N277 was identified. The genetic screening and mapping of N277, a mutant that exhibits insensitivity to C23-inhibition of ABA signaling, is presented in the following chapter.

Chapter One, in part, is being prepared for publication of the material, Kim TH; Ha T; Schroeder JI. The thesis author will be a co-author of this paper.
CHAPTER 2:

Isolation of an ABA/C23 signaling mutant
2.1 Introduction

The phytohormone abscisic acid (ABA) has been known to trigger many signaling pathways that regulate not only in response to abiotic stresses but during plant growth and development, including seed dormancy and germination as well (Finkelstein et al., 2002; Israelsson et al., 2006). ABA-response mutants are useful tools to study the ABA signal transduction pathway, which have revealed that ABA signaling requires protein kinases, phosphatases, and transcription factors (Finkelstein et al., 2002; Himmelbach et al., 2003). To understand the importance and complexities of ABA in plant life, the model organism *Arabidopsis thaliana* is employed. The first step of ABA responses requires the perception of ABA. The identification of ABA receptors is essential for the understanding of signaling pathways; however, information on ABA receptors has been limited. Research has suggested the presence of both extracellular and intracellular receptors in ABA signal transduction (Gilroy and Jones, 1994; Schwartz et al., 1994; Schwarz and Schroeder, 1998). Recently, candidate ABA receptors have been identified and reported. These include the Mg-chelatase H subunit GUN5, the G-protein coupled receptor GCR2, GTG1/GTG2, and PYR/PYL/RCAR (Shen et al., 2006; Liu et al., 2007; Pandey et al., 2009; Park et al., 2009). Mg-chelatase H subunit seems to play a role in stomatal movement (Shen et al., 2006). The ABA-binding protein ABAR is a Mg-chelatase H subunit that has a strong affinity and stereospecificity for ABA (Shen et al., 2006). Reduced expression or overexpression of *ABAR* leads to significant effects on the ABA signal in stomatal movement, seed germination, and post-germination growth (Shen et al., 2006). However, it is reported that the GUN5 homologous protein from
barley does not have an affinity to ABA and barley mutants of the that gene do not have stomatal phenotypes to ABA (Müller and Hansson, 2009).

Liu and colleagues have reported that a putative G protein-coupled receptors (GPCR) interact with the G protein α subunit GPA1 to mediate ABA responses. Over-expression of GCR2, a reported G protein-coupled plasma membrane receptor for ABA, results in ABA-hypersensitivity (Liu et al., 2007). However, other evidence contradicts the role of GCR2 as an ABA receptor. Molecular and genetic evidence reveal that neither GCR2 or its homologs, GCR2-LIKE 1 (GCL1), is required for ABA responses (Guo et al., 2007). Furthermore, the classification of GCR as a GPCR is questionable (Guo et al., 2008). Bioinformatics of GCR2 show that GCR2 does not even have a 7-transmembrane protein structure, a key characteristic of GPCRs (Guo et al., 2007).

GTG1 and GTG2 encode membrane proteins GPCR-type G proteins (Pandey et al., 2009). GTG1 interacts with GPA1 (G-protein subunit) and ABA binds to GTG1. The gpa1 mutants exhibited a partially reduced ABA response during stomatal opening (Wang et al., 2001). Hence, GTG1/GTG2 may represent membrane-localized ABA receptors that control ABA signaling through regulation of G-proteins.

Although more insight was gained about the perception of ABA, the relay between ABA perception and ABA signaling mechanisms has been unclear until recently. PYR/PYL/RCAR family proteins were identified as ABA binding proteins (Ma et al., 2009, Park et al., 2009). In Arabidopsis PYR/PYL/RCARs come from a family with 14 members that have structural similarities with class 10 pathogen-related proteins. ABA is shown to bind to RCAR1 and to antagonize PP2C activity in vitro (Ma et al., 2009). It has been shown that ABA binding to PYR/PYL/RCAR proteins induces
interaction with PP2C protein phosphatases. Complex formation among ABA, PYR/PYL/RCAR, and PP2C inhibits negative regulation of PP2Cs and initiate downstream ABA signaling.

Previous studies have shown an overlap between ABA and plant defense signaling. In addition to the information provided in the previous chapter, another source of evidence that shows an interconnection between ABA and pathogen resistance signaling comes from studying the transcription factor AtMYC2, initially identified as only a positive regulator of ABA signaling (Abe et al., 2003). When AtMYC2 is disrupted, the levels of basal and induced expression from JA- and ET-responsive defense genes were elevated (Anderson et al., 2004). Interestingly, the jasmonate-insensitive jin1 mutant showed that JINI is allelic to AtMYC2 (Lorenzo et al., 2004). Research suggests that AtMYC2 is a late point of convergence of ABA and JA signaling: it activates ABA-regulated gene expression and JA-mediated systemic responses to wounding, but inhibits JA-regulated genes that are involved in pathogen defense. Hence, the AtMYC2 knockout mutant, jin1, and the ABA-biosynthetic mutant aba2-1 were less susceptible to pathogen attack. (Anderson et al., 2004; Lorenzo et al., 2004).

Isolating a mutant is one of the best ways to discover new information and to uncover new genes that have critical roles in the area of interest. In order to isolate more genes mutant that have a specific function in ABA signal transduction and to dissect the signaling interaction between C23 and ABA signaling, forward genetic screens and subsequent positional or map-based cloning techniques can still be effective. Although it is laborious, isolation of mutants containing point mutations can provide direct
information regarding functional amino acids or domains generating specific protein activities.

The point mutation is induced in an Arabidopsis ecotype and crossed to another, generating a F2 mapping population that is used for mapping. If the mutation is recessive, the mapping population can be first screened for the phenotype to utilize only the plants with mutant phenotype for fine mapping. The genetic interval containing the mutation will then be narrowed by successively using and creating new molecular markers (Lukowitz et al., 2000). The molecular markers used to detect polymorphisms range from large deletions and rearrangements to single nucleotide polymorphisms, which include simple sequence length polymorphisms (SSLPs), cleaved amplified polymorphic sequences (CAPS), and derived CAPS (dCAPS) (Lukowitz et al., 2000; Brookes, 1999). Among the many Arabidopsis ecotypes that are divergent enough to allow the creation of molecular markers, the most commonly used pair for mapping is Landsberg erecta X Columbia, estimated to differ in four to 11 positions every 1,000 bp (Lukowitz et al., 2000; Chang et al., 1988; Konieczny and Ausubel, 1993; Hardtke et al., 1996). Furthermore, the sequences of Columbia and Landsberg erecta ecotypes have been determined, allowing for an extensive collection of molecular polymorphisms to be analyzed—making the Col X Ler pair a popular choice (Rounsley et al., 1999).

Once the chromosomal region with the mutation has been narrowed down, the mutated gene can be identified by several methods. The mutated gene can be identified by transforming overlapping fragments of wild-type DNA into the mutant to determine if the mutant trait can be restored to wildtype (Lukowitz et al., 2000). For gain-of-function
mutants with a dominant or semidominant inheritance, the dominant mutant allele can be transferred to wildtype to manifest the mutant phenotype (Leung et al., 1994). The complete DNA sequence in the genetic interval can also be scanned for changes that have created the mutation (Taylor, 1999).

In the previous chapter, more insight into the interactions between ABA and C23-mediated pathogen signaling in guard cells was gained. Results in the previous chapter have shown that the induction of defense signaling by Compound 23 (C23) inhibits ABA signal transduction. C23 is a synthetic chemical that inhibits ABA signaling and induces the expression of genes involved in the defense signaling pathway (Kim and Schroeder, unpublished). By observing the stomatal responses of the mutants defective in defense signaling under treatments with ABA and C23, results revealed that the crosstalk between the ABA and defense signaling involves EDS1, PAD4, RAR1, and SGT1B in a SA-independent manner. To uncover a new component that could be a convergent point between the two signaling pathways and to further understand the molecular mechanisms of how ABA signal transduction interact with defense signaling, forward genetic mutant screens using C23 and ABA were performed (Kim and Schroeder, unpublished).

In this thesis study, the N277 mutant is examined. The N277 mutation was induced by EMS to isolate mutants with insensitivity to C23-inhibition of ABA signaling. Identification of the N277 mutation will give more insight to how C23 can affect ABA signal transduction. As a first step of mutant cloning, a rough mapping of N277 is performed. The mutation of N277 is induced on a Columbia ecotype background and crossed to Landsberg erecta ecotype to create a N277xLer F2 mapping population. In hopes of isolating a new gene that could be critical in bridging the network behind ABA
and defense signaling, more information can be gained to understand the interactions between ABA signal transduction and plant defense signaling, and new discoveries can be applied to agriculture.

2.2 Materials and Methods

Plant Growth and Screening

An eppendorf tube of N277xLer F2 seeds were first sterilized in a solution with 70% ethanol and 0.05% SDS for 10 minutes with gentle agitation, followed by 3 washes of 100% ethanol. Ethanol was then removed from the eppendorf tube and the seeds were left to dry in the tube. Sterilized seeds of N277xLer F2, along with Col-0 expressing RAB18promoter-GFP as controls, were then plated on 0.5x Murashige and Skoog medium with 1% sucrose, 0.005% Mesh-Hydrate, and 0.08% agar, titrated to pH 5.8 with KOH. After 3 days of stratification at 4°C, plates were left to grow in a Conviron® growth chamber.

After ten days of growth or when the second set of rosette leaves emerge, plates are sprayed with 50µM ABA and 30µM C23. After 1 day of incubation, seedlings were screened for fluorescence under UV light. Seedlings with fluorescence were selected and transferred to soil to encourage further growth.

Genomic DNA extraction

Two leaves were extracted from each plant and transferred to a 1.5ml eppendorf tube. Leaves were crudely grinded with 150µl of extraction buffer (0.2 M Tris-HCl, pH
9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS). After centrifuging at 13,000 rpm for 6 minutes, the supernatant was transferred to a clean eppendorf tube and combined with equal volumes of isopropanol, followed by another round of centrifuging at 13,000 rpm for 12 minutes. Supernatant was then discarded and the pellet was washed with 700µl of 70% ethanol. After a quick centrifuge at 13,000 rpm for 3 minutes, the ethanol was discarded and the eppendorf was inverted upside to dry completely. The purified DNA was then resuspended in 100µl MQ water.

**PCR-based fine mapping for N277 locus and Synergel gels**

PCR was performed on the segregated N277 mutants in the N277xLer F2 population with selectable markers. An annealing temperature of 56°C and an elongation time of 30 sec at 72°C were used. A 1% agarose gel with 2% Synergel was used for gel electrophoresis. The customized gel was critical to distinguish the subtle differences in the PCR products (25 b.p. or less).

**Stomatal Closing Aperture Measurements**

The epidermis of rosette leaves from 4 to 5 weeks old plants were mounted on a cover-slip by gluing the epidermal side of the leaf onto a cover-slip and stripping away the mesophyll cells. Samples were then submerged in opening buffer (10 mM KCl, 7.5 mM iminodiacetic acid, 10 mM MES and pH 6.2 adjusted with KOH) for 2 hours under light with a fluence rate of 160 µmol m⁻² s⁻¹ at 20°C. After 2 hours of incubation, the opening buffer was then replaced with opening buffer containing 0, 1, or 10 µM ABA for
1 hour. Cover-slip samples were then transferred onto a microscope slide for viewing and measuring under a microscope.

Stomatal assays were performed in double blind experiments (plant identity and ABA concentrations were unknown). If necessary, pots with plants were kept overnight in high humidity with Saran-wrap before experiment.

2.3 Results

_N277 exhibits insensitivity to C23-inhibition of ABA signaling._

Screening of mutants induced by EMS revealed that N277 was a mutant that exhibited insensitivity to C23-inhibition of ABA signal transduction. Utilizing this C23-insensitive phenotype of N277, N277 was crossed to a RAB18-GFP promoter. N277 was created on a Columbia ecotype background and crossed to Landsberg _erecta_ to generate a N277xLer F2 mapping population. Selection of the segregated N277xLer F2 mutants containing the N277 mutation was based upon the presence of ABA-induced RAB18-GFP expression with the addition of ABA and C23 together.

In order to identify the mutated gene in N277, a map-based cloning strategy was employed. Previously, the N277 mutant was crossed to the Landsberg _erecta_ wildtype for generation of F2 seed population by Dr. Tae Houn Kim. To isolate segregated-F2 mutant plants, approximately 60 seeds of N277xLer F2 crosses and 2-3 seeds of transgenic lines expressing RAB18 promoter driven-GFP as wildtype controls were plated on a MS medium agar plate. Over 150 plates have been screened for this thesis study. Plates were placed under light and grown horizontally until the second set of true
leaves emerged. These seedlings were then sprayed with 50 μM ABA and 30 μM C23. The concentration of ABA was set at 50 μM, which is the concentration producing consistent ABA induction of the RAB18 promoter in 12% of WT. Upon UV light exposure after incubation with ABA and C23, some plates contained at most 2 seedlings with clear signals of green fluorescence (left, Figure 10), while others had more seedlings with green fluorescence (right, Figure 10). Unfortunately, the ABA and C23 treatments often produced inconsistent RAB18 expression patterns, which generated false positives and false negatives. This occurrence may have stemmed from the unnoticeably slight differences in experimental conditions for plant growth and stimuli treatments. Moreover, it could also be caused by the unstable nature of the recombinant RAB18 promoter driving GFP expression as a transgenic line. In order to minimize and dilute the effect of false positives, a large number of F2 mutant population was targeted to screen and selection of F2 mutants was restricted to the expected Mendelian ratio of two recessive genes (e.g. selecting only the brightest seedling out of 16).
Figure 10: Segregation of the N277 mutant phenotypes in N277xLer F2 population. GFP expression was examined by exposing plates with UV light after 1 day of ABA and C23 treatments. Segregated N277 F2 mutant seedlings showed insensitivity to C23 inhibition of the ABA-induced RAB18 expression. N277xLer F2 and Col-0 RAB18-GFP (corners) seedlings were sprayed with 50 μM ABA and 30 μM C23. Two seedlings (circles in the left panel) while more (circles in the right panel) were detected to exhibit effects of ABA signaling in spite of C23 inhibition.
To improve the accuracy of reporter-based phenotyping, I have also conducted GFP screening, based on guard cell expression of RAB18 promoter-driven GFP. Under normal environmental conditions, endogenous ABA levels were present and RAB18-GFP expression was observed in guard cells (Figure 11). In response to the exogenous application of ABA, the exogenous ABA further induced the RAB18-promoter in guard cells, resulting in a more elevated GFP expression in both the epidermis and mesophyl cells (Figure 11a). Correspondingly, only individual guard cells showed reduced fluorescence in the combined treatment of ABA and C23, while the fluorescence of the epidermis was significantly reduced in wildtype controls. This trend of fluorescence in the guard cells, but not in the other leaf tissues with the combined treatment of ABA and C23, was also observed in many segregated N277xLer F2 seedlings (Figure 11b), suggesting that these seedlings did not exhibit the N277 phenotype and did not contain the N277 locus. On the other hand, the N277xLer F2 segregated mutants that exhibited fluorescence in the mesophyll cells in addition to guard cells in spite of C23 inhibition (Figure 11c) revealed the presence of the original N277 mutation in these seedlings. These seedlings were selected and transferred to soil for future genotyping. Although it took more labor and time, examination of guard cells under the fluorescence microscope gave more consistent and distinguishable ABA-induced RAB18 expression patterns. Over 1,000 seeds were screened and approximately 60 selected seedlings were transferred to soil to encourage further growth for genomic DNA extraction.

The observation of fluorescence in both guard cells and the mesophyll cells with ABA and C23 in N277xLer F2 mutants was compared to the parent N277 mutant to confirm the validity of this guard cell expression phenotype. Indeed, the N277 mutant
maintained the RAB18 promoter-driven GFP expression in combined treatment of ABA and C23 as seen by the fluorescence in the mesophyll cells (Figure 12b right).
Figure 11: Segregation of N277 mutant phenotypes in N277xLer F2 population. GFP expression of guard cells was examined with confocal microscopy after 1 day of ABA and C23 treatments on plates. Stomata of segregated N277 phenotype in N277xLer F2 mutant exhibit insensitivity to C23 inhibition of ABA-induced RAB18 expression. a) Comparing stomata of wildtype controls between treatments of 50 μM ABA (left) and 50 μM ABA+30 μM C23 (right) show suppressed ABA-induced RAB18 expression. b) Comparing stomata of the wildtype progeny segregated from N277xLer F2 populations between treatments of 50 μM ABA (left) and 50 μM ABA+30 μM C23 (right) reveal sensitivity to C23 inhibition of ABA-induced RAB18 expression by C23 inhibition. c) Comparing stomata of selected N277xLer F2 mutants reveal insensitivity to C23 inhibition of ABA-induced RAB18 expression.
Figure 12: Confocal images of leaf epidermis reveal insensitivity to C23 inhibition of ABA-induced RAB18 expression in the parent N277 mutant. Whole seedlings grown on MS liquid medium were treated with water, 50 µM ABA, or combined 50 µM ABA + 30 µM C23 for 5 hours under light. a) Comparison of transgenic control leaf epidermis between treatments of water (left), 50 µM ABA (middle), and 50 µM ABA + 30 µM C23 (right) demonstrates inhibition of ABA-induced RAB18 expression by C23. b) N277 mutant phenotype of leaf epidermis between treatments of water (left), 50 µM ABA (middle), and 50 µM ABA + 30 µM C23 (right) reveal insensitivity to C23 inhibition of ABA-induced RAB18 expression.
Genotyping N277xLer F2 mutant plants with selectable markers for rough mapping of the mutant gene.

The original N277 mutation was induced in a Columbia background and crossed with Landsberg erecta for map-based cloning. Molecular markers of simple sequence length polymorphisms (SSLPs) that covered the representative regions of the entire genome of Arabidopsis (Table 1) were used for rough mapping of a mutation (Lukowitz et al., 2000). Quantifying the linkage scores involved counting the genotypes that resulted in each molecular marker. A Columbia marker is designated with a score of 2 for Columbia, a Landsberg marker is designated with a score 2 for Landsberg, and a hybrid or a heterozygous marker is designated with a score of 1 for both Columbia and Landsberg. Hence, results that contained 6 Columbia markers, 8 hybrid markers, and 1 Landsberg marker (Figure 13a) will produce a Col-Ler raw score of 20-10 or a Col:Ler ratio of 2:1 (Figure 13b). This raw score, a higher score for Columbia than for Landsberg, represents a bias towards Columbia linkage. Therefore, a high score for Columbia, compared to a low Landsberg score, represents a Columbia linkage. The raw scores of all selectable markers and their corresponding Col:Ler ratios experimented in the selected N277xLer F2 mutants are represented in Table 2.

The linkage scores, Col-Ler raw scores and Col:Ler ratios, suggest the N277 locus to be most likely located in chromosome 2; however, there is also evidence of linkage in chromosome 3, 4, and 5 (Table 2). Most of the regions in chromosome 2 display a heavy bias towards Columbia linkage, based upon its high Columbia raw scores and ratio compared to that of Landsberg (Table 2). Additionally, the top of chromosome 3 and 4, and the bottom of chromosome 5 display a bias towards Columbia linkage as well. This
initial rough mapping of N277 suggests many possible locations for the gene locus of N277. Therefore, to provide a more specific location of where the N277 locus is located, additional mutant screening with alternative methods and further rough mapping will be needed.
Table 1: List of all molecular markers (SSLPs) used for rough mapping.

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<th>Kb Pos.</th>
<th>Primer sequence</th>
<th>Polymorph.</th>
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Figure 13: An example of genotyping a sample of the selected N277xLer F2 mutants to determine any linkage to Columbia-derived markers. a) PCR on genomic DNA from wildtype Col and Ler (1 and 2) and selected N277xLer F2 mutants (3-17) with primers located at 264 kb of chromosome 2 reveal a partial linkage to the Columbia marker. b) The genotypes of the mutant samples are quantified to represent any linkage to Columbia. In this example, a slight bias in the linkage towards the Columbia marker suggest that the N277 locus may be in chromosome 2.
Table 2: The cumulative linkage scores of all selectable markers suggest the N277 gene locus to be most likely located in Chromosome 2.

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Characterization of N277 reveals a partial insensitivity to ABA in ABA-induced stomatal closing experiments.

To gain further insight into the mutated gene in N277, an ABA response of the N277 mutant was characterized through ABA-induced stomatal closing experiments. Figure 14 represents the average of 3 double-blinded experiments of ABA-induced stomatal closing experiments. By analyzing the stomatal apertures with 1 μM ABA, the stomatal apertures of N277 were less closed than those of wildtype. In the treatment with 10 μM ABA, the stomatal apertures of N277 were also less closed than the stomatal apertures of wildtype (Figure 14). The ABA-induced stomatal closing experiment revealed that N277 exhibits a partial insensitivity to ABA, compared to wildtype Col-0 expressing RAB18 promoter-driven GFP.
Figure 14: Measurements of ABA-induced stomatal closing aperture reveal moderate ABA insensitivity of N277. Comparison of stomatal apertures between wildtype (Col-0 RAB18-GFP) and N277 with treatments 0, 1, and 10 µM ABA using the Stripping Method. N=3 experiments, 30 stomata per treatment.
2.4 Discussion

Mutant screens and genetic cloning have efficiently served to identify new genes. One of the advantages of genetic approaches to study signal transduction is that *in planta* function of newly identified genes can be examined right away by analyzing mutant phenotypes. Hence, many genes that are directly involved in the ABA and defense signaling pathways have been isolated by forward genetic screening methods. Although past decades of research have revealed the function of identified genes and mechanisms of ABA and defense signaling individually, however, the details of interactions between these two signal transduction pathways are still not understood. In this thesis study, the N277 mutant is isolated as an attempt to discover a new component that has a role in both ABA and defense signaling and to further understand the dynamic interactions between the two major stress signaling pathways.

*Complications of isolating N277 suggest the need for alternative methods.*

The N277 mutant was originally isolated by mutant screens for altered C23-response mutants from EMS (ethyl methanesulfonate)-mutagenized population (Kim and Schroeder, unpublished). To identify the mutated gene, map-based cloning of N277 was employed. The reduced sensitivity to C23-inhibition of ABA signaling phenotype was utilized in selecting the segregated N277 mutant phenotype in the N277xLer F2 population. Selection of the segregated N277 mutants in the N277xLer F2 population was based upon RAB18-GFP expression induced by ABA, even in the presence of C23 inhibition of ABA signaling (Figure 10). Screening of the segregated N277 mutants in the N277xLer F2 population was not as efficient and reliable as predicted because false
positives were often produced or very few N277 mutants were selected. This occurrence may have stemmed from the slight differences in the conditions for plant growth and stimuli treatments, as well as inconsistent ABA induction especially during a season of drought. Hence, more seedlings of N277xLer F2 need to be screened to minimize the misleading effects of false positives as a focus for future research. In addition, the RAB18-GFP promoter that was produced on a Columbia background may also have obscured the data of linkage scores since it itself was another site of Columbia linkage in addition to the N277 locus.

According to the linkage scores of Table 2, the selectable markers with the highest Columbia score, compared to the Landsberg score, were the selectable markers located in chromosome 2, suggesting that the possible location of the N277 locus may be in chromosome 2. However, to provide a more specific location of where the N277 locus is located, further rough mapping of N277 and the use of alternative methods to isolate N277 will be needed.

There are many promising ways to improve the isolation of N277, all of which can serve as a focus for future research. First, the mapping population can be produced using an ecotype other than Landsberg erecta. Although having the entire genome of Landsberg erecta was beneficial, the N277xLer F2 population suggested that Landsberg erecta may not have been the optimal choice to isolate N277. The accuracy in selecting the N277xLer F2 mutants with the N277 phenotype was limited to the success of ABA-induced RAB18-GFP expression, which was inconsistent throughout the screening of N277xLer F2 mutants. Thus, the use of another promoter, such as RD29b-GUS, could potentially produce a stronger expression than RAB18-GFP with ABA induction,
allowing the selection of N277 mutants in the mapping population to be more successful. Last, there is another phenotype that could be utilized in the selection of N277 mutants. The C23-insensitive phenotype of N277 suggests that the induction of pathogen defense signaling is weakened or impaired in N277, indicating that N277 has increased susceptibility to a pathogen invasion. This increased susceptibility to pathogens can be serve as an alternate phenotype to screen for the N277 mutants in the mapping population. Although the isolation of N277 through the N277xLer F2 population with the RAB18-GFP promoter may not have provided a specific locus for the N277 gene, there are other alternatives to ensure a successful isolation of N277.

**The C23-insensitive N277 mutant displays partial insensitivity to ABA signaling**

To explore whether N277 has an altered response in ABA signal transduction in guard cells, ABA-induced stomatal closure assays were performed on N277 (Figure 14). When comparing the apertures of N277 to Col-0 wildtype in 1 μM and 10 μM ABA treatments, the stomatal apertures of N277 were not as closed as those of wildtype. Hence, N277 exhibits partial insensitivity to ABA signaling, suggesting that the N277 gene plays a role in ABA signaling. Since N277 showed a partial insensitivity to ABA, the N277 gene may function as redundantly with other homologous genes in Arabidopsis. As a focus for future research, stomatal responses of N277 can be analyzed under treatments with both ABA and C23 to determine if the partial lack of sensitivity to ABA signaling in N277 can be recovered by the induction of defense signaling through the application of C23 and to understand the dynamics between ABA and defense signaling on the stomatal level.
Research with N277 thus far have directed its function to be present in both ABA and defense signaling, because N277 displayed reduced sensitivities to both ABA and C23 treatments. ABA is involved in responses to drought, while the phytohormones, salicylic acid (SA) and jasmonic acid (JA) are involved in plant defense signaling (Finkelstein et al., 2002; Fujita et al., 2006). Continuing from the previous chapter revealing the crosstalk between ABA and C23-mediated defense signaling to involve EDS1, PAD4, RAR1, and SGT1B on a SA-independent level, this chapter showed that N277 could be another component critical in the interactions between the two signaling pathways. Studies related to the inhibition or lack of ABA signaling have revealed a correlation between elevated ABA levels and increased susceptibility to pathogen attack; others have unveiled the antagonistic relationship between ABA and defense signaling (Mauch-Mani and Mauch, 2005; Anderson et al., 2004). The antagonistic relationship indicates that plants maintain a balance between the opposing forces of the signaling pathways to reach a state at which plant immunity is at an optimal level without endangering plant growth and development, which are under ABA regulation. Understanding the molecular mechanisms of how ABA signal transduction communicates with defense signaling against pathogen invasions will be helpful to enhance plant immunity without harming crop yield.
APPENDIX:

ABA-induced stomatal closing of triple pp2ca mutant
A.1 Introduction

The stomata, tiny pores formed by a pair of guard cells, open and close to regulate of CO$_2$ uptake and transpirational water loss. In response to a water deficit, the phytohormone abscisic acid (ABA) stimulates a signaling cascade that leads to the closure of stomatal pores. Protein kinases and phosphatases have been discovered to control protein phosphorylation and dephosphorylation events in ABA signaling (Leung and Giraudat, 1998; Finkelstein et al., 2002).

Type 2C protein phosphatases (PP2Cs), transcriptionally up-regulated by ABA, negatively regulate ABA signaling (Finkelstein et al., 2002; Christmann et al., 2006). With such a large population of PP2Cs with overlapping functions, their functional redundancies imply the presence of a complex PP2C-substrate network for the intricate ABA signaling pathway (Hirayama and Shinozaki, 2007). The single reduction/loss-of-function alleles from ABA-INSENSITIVE (ABI1), ABA-INSENSITIVE2 (ABI2), and HYPERSENSITIVE TO ABA 1 (HAB1) have varying degrees in the strength of ABA phenotype; likewise, double mutants $hab1$-1 $abi1$-2 and $hab1$-1 $abi1$-3 produce a greater response to ABA than the single mutants alone (Merlot et al., 2001; Saez et al., 2006). The double mutants were strongly hypersensitive to ABA in growth assays and stomatal closure, suggesting a cooperative negative regulation of ABA signaling (Saez et al., 2006). Hence, the fine tuning of ABA signaling can be accomplished through the combined action of PP2Cs (Saez et al., 2006). The following is a stomatal closing experiment performed on the Arabidopsis $hab1$-1 $abi1$-2 pp2ca-1 mutant to further investigate cooperative negative regulation of ABA signaling in triple mutants.
A.2 Materials and Methods

For stripping stomatal assays, the epidermis of rosette leaves from 3 to 4 week old plants were mounted on a cover-slip by gluing the epidermal side of the leaf onto a cover-slip and stripping away the mesophyll cells. Samples were then submerged in opening buffer (10 mM KCl, 7.5 mM iminodiacetic acid, 10 mM MES and pH 6.2 adjusted with KOH) for 2 hours under light with a fluence rate of 160 µmol m$^{-2}$ s$^{-1}$ at 20°C. After 2 hours of incubation, the opening buffer was then replaced with opening buffer containing 0, 1, or 10 µM ABA for 1 hour. Cover-slip samples were then transferred onto a microscope slide for viewing and measuring under a microscope.

Stomatal assays were performed in double blind experiments (plant identity and ABA concentrations were unknown. If necessary, pots with plants were kept overnight in high humidity with Saran-wrap before experiment.

A.3 Results

The hab1-1 abi1-2 pp2ca-1 mutant reveals moderate ABA hypersensitivity

The Arabidopsis hab1-1 abi1-2 pp2ca-1 mutant was isolated by Dr. Pedro Rodriguez, which showed ABA-hypersensitivity in germination and root growth. An ABA-induced stomatal closing experiment was performed on hab1-1 abi1-2 pp2ca-1 to characterize the ABA-hypersensitivity phenotype by observing the responses of the guard cells. Although the experiment was performed in a relatively dry season, results reveal that hab1-1 abi1-2 pp2ca-1 exhibit a moderate hypersensitivity to ABA, compared to the Col-0 wildtype response (Figure 15).
Figure 15: Measurements of ABA-induced stomatal closing aperture of hab1-1 abi1-2 pp2ca-1 reveals moderate ABA hypersensitivity. Comparison of stomatal apertures between Col-0 wildtype and hab1-1 abi1-2 pp2ca-1 with treatments 0, 10, and 100 nM ABA using the Stripping Method. N=3 experiments, 30 stomata per treatment.
A.4 Discussion

Previous studies with *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants have shown the additive affects of double mutants. The double mutants surmount to a greater hypersensitive response to ABA than the single mutants alone, suggesting a cooperative negative regulation of ABA signaling (Merlot et al., 2001; Saez et al., 2006). The cooperative functions of ABA signaling was further investigated using the *hab1-1 abi1-2 pp2ca-1* triple mutant. The results of ABA-induced stomatal closing experiment confirmed this trend of increasing strength in hypersensitivity to ABA, suggesting the highly probable presence of a cooperative negative regulation of ABA signaling.
REFERENCES

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