The undersigned, appointed by the dean of the Graduate School, have examined
the dissertation entitled

**INVESTIGATING THE ROLES AND FUNCTIONS OF PLASMA MEMBRANE E3 LIGASES AND MKP1 IN PLANT IMMUNITY**

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ABSTRACT

When a plant encounters a pathogen, a complex set of responses occurs on both sides of the interaction. In a process known as pattern-triggered immunity (PTI), the plant uses plasma membrane (PM)-localized proteins, or pattern recognition receptors (PRRs), to perceive the presence of a threat by recognition of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs), which are non-self molecules endogenous to the pathogen but not the plant. After perception, these PRRs initiate signaling events leading to canonical PTI responses such as activation of the MAP kinase cascade, production of reactive oxygen species (ROS) and regulation of defense-related transcripts. At the same time, the pathogen recognizes a potential host through plant-derived metabolites, which induces its type-III secretion system (T3SS) to inject effector proteins into the plant cell to make nutrient acquisition easier. Due to the effector proteins’ key role in virulence, a goal of the plant during these early stages of the interaction is to restrict the delivery of effectors into the plant cell. Decreasing the abundance of T3SS-inducing metabolites from the extracellular space, essentially masking its presence from the pathogen, is sufficient to drastically reduce the number of effectors translocated into the cell, conferring resistance to the plant. Previous work in the lab established a knockout of MAP Kinase Phosphatase 1 (mkp1) as a model for studying this layer of PTI. mkp1 plants restrict effector delivery into the plant, resulting in greater resistance than in wild type plants. This resistance is due to a basal decrease in extracellular T3SS-inducing metabolites compared to wild
type. A key question remains as to how the plant is able to modulate the accumulation of T3SS-inducing metabolites. The work in this dissertation establishes the PM-localized E3 ligase, ATL6, as a key positive regulator of PTI, playing an important role in PTI-associated resistance and effector delivery restriction, while not impacting other canonical PTI phenotypes. In addition, using an atl6 mkp1 double mutant, I establish that ATL6 is genetically linked to MKP1, suggesting a specific role in the regulation of T3SS-inducing metabolites and effector delivery restriction. I also identify another E3 ligase, ATL31, as a positive regulator of PTI, with atl31 knockouts showing phenotypes similar to atl6 plants. Together, these mutants define novel regulators of plant immune responses that are required for the resistance observed in mkp1 mutants.

When effectors are translocated into the plant cell, they inhibit defense responses by targeting a spectrum of proteins involved in plant immunity. However, plants have evolved another layer of defense called effector-triggered immunity (ETI), which is activated when a class of intracellular receptors (R genes) recognize the presence or the activity of the effector. Recognition results in a more robust defense response, often leading to cell death. Due to the decrease of effectors being delivered to mkp1 plants, we hypothesized that mkp1 plants may be partially compromised in their ability to mount an ETI response because there are fewer effectors to trigger ETI. Surprisingly, this work shows the opposite result: mkp1 plants are more resistant to ETI-triggering bacterial strains, establishing MKP1 as a negative regulator of ETI. In addition, further work with higher order
mutations shows that MKP1 acts in a distinct pathway during ETI compared to PTI. Thus, this work identifies a new role for MKP1 in ETI and suggests the existence of a previously undescribed pathway.
Pattern-triggered immunity vs. pathogen virulence

Plants have evolved a set of complex mechanisms to perceive, respond, and adapt to bacterial pathogens. When a plant encounters a bacterium, it utilizes plasma membrane (PM)-localized proteins known as pattern recognition receptors (PRRs) to perceive the presence of non-self molecules known as pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) to trigger pattern-triggered immunity (PTI) (Macho and Zipfel, 2014; Newman et al., 2013; Bigeard et al., 2015). These molecules are endogenous to the pathogen but not the plant. Examples include conserved peptides from bacterial flagellin, flg22, which is recognized by its cognate receptor FLAGELLIN SENSING 2 (FLS2) (Felix et al., 1999; Gómez-Gómez and Boller, 2000) and from Elongation Factor-Tu, elf18/26 (depending on the length of the peptide), which is recognized by the EF-Tu receptor (EFR) (Zipfel et al., 2006). After initial perception, the kinase domains of the PRRs and their coreceptor BAK1 are activated, leading to a coordinated set of downstream responses (Chinchilla et al., 2006; Heese et al., 2007; Chinchilla et al., 2007). Included in these responses are ligand-induced endocytosis and degradation of the PRR to modulate activity (Robatzek et al., 2006; Smith et al., 2014), production and accumulation of reactive oxygen species (ROS) (Pogány et
al., 2009; Apel and Hirt, 2004), activation of MAP Kinases (MAPKs) (Colcombet and Hirt, 2008), and regulation of defense-related transcript accumulation (Boller and Felix, 2009). Plants lacking proteins required for perception and/or initiation of responses are unable to mount a PTI response and defend themselves effectively against an invading pathogen (Zipfel et al., 2004; Lacombe et al., 2010; Miya et al., 2007).

A key aspect of PTI signaling is the activation of MAPK cascades (Colcombet and Hirt, 2008). MAPKs play a wide range of roles in the plant and serve as an important signaling hub. The cascades include MAPK Kinase Kinases (MAP3Ks) which are activated downstream of the PRRs. MAP3Ks phosphorylate MAPK Kinases (MAPKKs), in turn phosphorylating the terminal MAPKs at a Thr-X-Tyr activation loop (Bartels et al., 2010). The *Arabidopsis* genome consists of 20 MAPKs and 10 MAPKKs which are used to amplify, transduce, and integrate multiple signaling responses (Ichimura et al., 2002; Hamel et al., 2006). MPK6, MPK3, and MPK4 are the three MAPKs shown to be activated after PAMP treatment, with MPK6 and MPK3 most closely associated with a PTI response. MPK6 and MPK3 appear to be at least partially redundant and are phosphorylated by the MAPKKs MKK4 and MKK5 (Asai et al., 2002; Nühse et al., 2000).

Once the signal is activated, it also has to be attenuated to ensure proper intensity and duration of the response. Phosphatases play a key role in negatively regulating the MAPKs (Owens and Keyse, 2007; Dickinson and Keyse, 2006). Dephosphorylation of either the threonine or tyrosine residues partially
deactivates the MAPK, while dephosphorylation of both is required for complete deactivation. Protein tyrosine phosphatases and serine-threonine phosphatases have both been shown to dephosphorylate MAPKs in vivo (Dickinson and Keyse, 2006). Because dual-specificity protein phosphatases can dephosphorylate both the threonine and tyrosine residues, they are an important category of negative regulators for the MPKs. MAPK Phosphatases (MKPs) are a family of dual-specificity protein phosphatases that are able to dephosphorylate both threonine and tyrosine residues (Camps et al., 2000; Bartels et al., 2010; Luan, 2003; Jiang et al., 2018). MAPK Phosphatase 1 (MKP1) is a key negative regulator of plant immunity. Previously, our lab showed that defense responses are hyperactivated after PAMP elicitation in MKP1 knockout plants (mkp1), including activation of MPK6 and MPK3 (Anderson et al., 2011). In addition, mkp1 plants are more resistant to bacterial infection. MPK6 is required for the increased PAMP responses and resistance in mkp1 plants, indicating a key genetic link between MKP1 and MPK6 (Anderson et al., 2011).

While a plant attempts to perceive and respond to an invading pathogen, the bacteria attempt to identify potential hosts to gain nutrients. To maintain homeostasis, the plant is constantly taking up and secreting metabolites. Pseudomonas syringae pathovar tomato (Pst DC3000) has evolved a mechanism to perceive a subset of these metabolites to recognize a potential host (Anderson et al., 2014; Yan et al., 2019; O’Malley et al., 2019). This perception leads to the activation of the type-III secretion system, a molecular syringe the bacteria use to
inject effector proteins into the plant cell to inhibit defense responses in the plant (Collmer et al., 2009; Hogenhout et al., 2009; Cunnac et al., 2009). Some of these effectors, such as AvrPto and AvrPtoB, actively target proteins involved in PAMP perception in the plant to prevent manifestation of a full PTI response (Xiang et al., 2008; Cheng et al., 2011; Gimenez-Ibanez et al., 2009; Göhre et al., 2008). Without these effectors, the pathogen is impaired in its ability to infect, as the plant is able to mount a more robust PTI response (Zipfel et al., 2004; Lin and Martin, 2007; Cunnac et al., 2011).

Due to the importance of effectors to a pathogen’s virulence strategy, an important aspect of plant defense is to restrict effector delivery into the plant cell. Although PTI induction leads to a host of responses, one of the most direct mechanisms of resistance is the restriction of effector delivery. PAMP pretreatment leads to a decrease in effector translocation into the plant cell (Crabill et al., 2010; Oh et al., 2010), and this restriction occurs in less than 2 hours. Previously, our lab discovered that mkp1 knockout plants mimic a PAMP-elicited plant by restricting both pathogen growth and effector delivery (Anderson et al., 2011; Anderson et al., 2014). This increased resistance is due to a decrease in the accumulation of extracellular T3SS-inducing metabolites (Anderson et al., 2014). Exogenous addition of a subset of these metabolites to mkp1 plants or PAMP-elicited wild type plants is sufficient to suppress the resistance phenotype, underlying the importance of this metabolite decrease in PTI. A key question remaining, however, is how the plant is able to modulate the accumulation of these
metabolites in the extracellular space. A potential hypothesis is that key metabolite transporters are regulated during a PTI response, most likely through removing the transporters from the PM and, therefore, decreasing extracellular T3SS-inducing metabolites. A major goal of this work is to address this question and determine how the plant alters the accumulation of T3SS-inducing metabolites.

**Effector-triggered immunity**

When the plant encounters a pathogen, it attempts to use PTI to prevent the injection of effector proteins, thereby avoiding suppression of defense responses and promotion of disease symptoms. However, if the bacteria’s activation of their T3SS is sufficiently rapid or robust, they are still able to inject sufficient effector proteins into the plant cell to inhibit PTI, promote nutrient acquisition, and allow further colonization of the plant (Collmer et al., 2009; Hogenhout et al., 2009). When the pathogen is able to overcome PTI to inject effectors, the plant has evolved another layer of defense known as effector-triggered immunity (ETI) to compensate (Dodds and Rathjen, 2010). Plants utilize resistance (R) proteins, a diverse set of related proteins that recognize either the activity of or the effector proteins themselves to re-initiate a strong form of resistance that can result in programmed cell death (Dangl and Jones, 2001; Cui et al., 2015). For example, the effector AvrRpm1 leads to the phosphorylation of the *Arabidopsis* protein RIN4 at the PM, which is then recognized by RPM1, leading to a robust ETI defense response (Mackey et al., 2002; Boyes et al., 1998). The presence of another effector,
AvrRps4, results in the accumulation of RPS4 in the nucleus, also leading to an ETI response (Wirthmueller et al., 2007). While effector delivery into the plant cell can result in inhibition of some defense responses, if the plant has the cognate R gene to an injected effector, it is able to mount a more robust defense response to overcome the presence of the effector proteins.

Because removing MKP1 from the plant leads to increased resistance and restriction of effector delivery, we hypothesized that perhaps a tradeoff for this positive trait is an impairment in inducing an ETI response. Because fewer effectors are being translocated into the plant cell, one would presume that the decreased number of effector proteins to be recognized by R genes could result in a weakened ETI response. Another major goal of this work is to investigate whether removing MKP1 from the plant results in deleterious effects in the ETI response, which could provide an explanation for why this apparently beneficial mutation in MKP1 has not been observed in nature.

**Research goals and major findings**

In this dissertation, I utilized the interaction between *Arabidopsis thaliana* and *Pseudomonas syringae* to investigate both the mechanism by which plants limit the amount of extracellular T3SS-inducing metabolites during a PTI response as well as the potential role of MKP1 in ETI. The experimental results are divided into the following chapters:
1) Chapter 2: Establishing ATL6 as a positive regulator of PTI functioning in the MKP1-dependent pathway

2) Chapter 3: MKP1 functions as a negative regulator of effector-triggered immunity

3) Chapter 4: Elucidating other members of the MKP1-dependent PTI pathway

In chapter 2, I identify the PM-localized E3 ligase Arabidopsis Tóxicos Levadura 6 (ATL6) as a novel positive regulator of PTI. Using knockout mutants, I show that atl6 plants are compromised in their ability to mount an effective PTI response. In addition, atl6 plants are unable to effectively restrict effector delivery after PAMP elicitation. Surprisingly, although atl6 plants are clearly unable to induce PTI, several canonical PTI molecular responses are unaltered, indicating a specific, downstream role for ATL6 in PTI. Due to its ability to restrict effector delivery, I hypothesized that ATL6 may function with MKP1 during a PTI. Utilizing an atl6 mkp1 double mutant, I confirm that resistance seen in mkp1 plants requires ATL6, genetically linking them in a pathway.

In chapter 3, I investigate whether removal of MKP1 leads to a compromised ETI response in knockout plants. Surprisingly, instead of a negative tradeoff, I show that mkp1 plants are more resistant to ETI-triggering strains of Pst DC3000. In addition, utilizing informative double mutants, I show that the enhanced ETI in mkp1 does not require MPK6 and ATL6, the two proteins known
to be required for the enhanced PTI resistance in \textit{mkp1}. This result suggests that MKP1 functions in a novel, undescribed pathway during an ETI response. I also test a series of other pathway candidates based on known or hypothesized interacting partners with MKP1, but none of them are required for \textit{mkp1} resistance to ETI-triggering strains of \textit{Pst} DC3000. Surprisingly, despite the resistance to \textit{Pst} DC3000 seen in \textit{mkp1} plants being independent of salicylic (SA) (Ying Wan thesis), the resistance seen in \textit{mkp1} plants to ETI-triggering strains of \textit{Pst} DC3000 requires SA production, as an \textit{m kp1 sid2} mutant suppresses the enhanced ETI in \textit{m kp1}.

In chapter 4, I begin an investigation into other potential members of the PTI signaling pathway being mis-regulated in the absence of MKP1. After showing that ATL6 is required for a full PTI response, I identify ATL31, its most similar family member, as another positive regulator of PTI. Using \textit{atl31} knockout plants and a similar set of experiments as previously described for \textit{atl6}, I show that ATL31 appears to have a very similar function as ATL6 in PTI. I also begin to investigate the CBL-CIPK signaling network’s potential role in PTI. CIPK14 has been shown to phosphorylate ATL31 \textit{in vitro}, suggesting that this family of kinases may play a role upstream of the ATLS in PTI (Yasuda \textit{et al.}, 2017). Utilizing a quintuple \textit{cbl} knockout that eliminates all CIPK activity at the PM, I show that \textit{cbl} plants phenocopy the \textit{atl} knockouts, indicating the ATLS and the CBL-CIPK network may function linearly in PTI.

In Appendix A, I briefly outline the generation of some genetic tools I completed during my dissertation research. First, I generated CRISPR-Cas9
constructs to knockout expression of ATL6 and ATL31 from plants in the Ws background. I also generated phospho-null and phospho-mimic constructs of ATL6 that will be used to help determine the role of ATL6 phosphorylation in the function of this protein.

Finally, I finish with a discussion of the major findings and conclusions of the dissertation and the future directions. Chapters 2 and 3 are drafts of manuscripts that are almost ready for publication. Only a few remaining experiments are needed and are currently being carried out by another graduate student. Chapter 4, as well as some of the genetic tools shown in Appendix A, lay the groundwork for part of that same graduate student’s dissertation research.
CHAPTER 2

Establishing ATL6 as a positive regulator of PTI functioning in the MKP1-dependent pathway

This chapter is a draft of a manuscript that will soon be submitted after a few final experiments are finished in the lab.

Introduction

When a plant encounters a pathogen, the plant initiates a highly regulated set of processes to restrict pathogen growth. The first layer of defense is pattern-triggered immunity (PTI). During PTI, plasma membrane (PM)-localized pattern recognition receptors (PRRs) recognize non-self molecules known as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively), which are intrinsic to the pathogen but not present in the host. In *Arabidopsis thaliana*, these PRRs include Flagellin Sensing 2 (FLS2) and EF-Tu Receptor (EFR) that recognize conserved peptides from bacterial flagellin (flg22) and EF-Tu (elf18/26), (Felix *et al.*, 1999; Zipfel *et al.*, 2006). After this initial perception, several discrete downstream pathways are activated, including the production of reactive oxygen species (ROS), the activation of the MAPKs, and the accumulation of defense-related gene transcripts (Boller and Felix, 2009). Plants lacking proteins required for the initial PTI response are not able to mount a robust defense response,
rendering it more susceptible to bacterial infection (Zipfel et al., 2004; Lacombe et al., 2010).

While the plant attempts to prevent infection, bacterial pathogens such as *Pseudomonas syringae* pathovar tomato DC3000 (*Pst* DC3000) attempt to colonize the plant by utilizing a type III secretion system (T3SS) to inject effector proteins into the plant cell to inhibit PTI defenses (Collmer et al., 2009; Hogenhout et al., 2009). For example, the effector proteins AvrPto and AvrPtoB target the PTI perception mechanism to suppress resistance (Xiang et al., 2008; Cheng et al., 2011; Gimenez-Ibanez et al., 2009; Göhre et al., 2008). Bacteria lacking these two effectors are less virulent on the plant, further demonstrating both the importance of an effective PTI response to restrict bacterial growth and the contribution of effectors to suppress these activities (Zipfel et al., 2004; Lin and Martin, 2007; Cunnac et al., 2011).

Due to the importance of effectors to the virulence of the pathogen, plants attempt to restrict their delivery into the plant cell; and inducing PTI with a PAMP treatment has been shown to restrict effector delivery (Crabill et al., 2010; Oh et al., 2010). The mechanism by which this occurs appears to be through manipulation of the plant-produced chemicals, such as citric acid and aspartic acid, required by the pathogens to induce their T3SS (Anderson et al., 2014; Yan et al., 2019; O’Malley et al., 2019) Plants lacking a negative regulator of PTI responses, MAP Kinase Phosphatase 1 (MKP1), are more resistant to bacterial infection due to a decrease in the extracellular abundance of a subset of these T3SS-inducing metabolites.
(Anderson et al., 2011; Anderson et al., 2014). Adding back these metabolites has been shown to restore the bacteria’s ability to inject effector proteins both in \textit{mkp1} knockout plants and after inducing PTI (Anderson et al., 2014), indicating that \textit{mkp1} plants mimic a PAMP-treated plant. An important question that remains, however, is how plants modulate the extracellular accumulation of these bioactive metabolites. Potential mechanisms for this restriction could be either changes in the activity or changes in the abundance of transporters of these metabolites in the plasma membrane proteome.

E3 Ubiquitin Ligases have been shown to play a number of roles in regulating PTI activity in \textit{Arabidopsis thaliana} (Zhou and Zeng, 2017). The PUB family of U-box E3 ligases has been extensively studied, in particular PUB12 and PUB13, which directly ubiquitinate FLS2 in a flg22-dependent manner, leading to its endocytosis and degradation (Lu et al., 2011). \textit{pub12} and \textit{pub13} mutants showed hyperaccumulation of ROS and defense-related transcripts after flg22 elicitation, indicating aberrant signaling (Lu et al., 2011). In addition, a \textit{pub22/23/24} triple mutant shows increased ROS production and defense-related transcript accumulation after PAMP treatment (Trujillo et al., 2008; Stegmann et al., 2012); and \textit{pire} knockout mutants, which lack an E3 ligase that leads to the degradation of RBOHD, the NADPH oxidase required for ROS production after PAMP elicitation, also have increased ROS accumulation after flg22 treatment, as well as increased resistance to bacterial infection (Lee et al., 2020). Another component of early PTI signaling is BIK1, a cytosolic kinase that is a component of the FLS2
receptor complex (Lu et al., 2010; Li et al., 2014). The E3 ligases RHA3A and RHA3B monoubiquitinate BIK1, causing BIK1 to be released from the receptor complex and internalized into endocytic compartments. ami-RNA-RHA3A/B lines also show decreased ROS production and increased susceptibility to Pst DC3000 (Ma et al., 2020). A commonality amongst all these E3 Ligases functioning in PTI is that their known targets are almost exclusively early signaling components, altering early molecular responses such as ROS accumulation and defense-related transcript accumulation.

The Arabidopsis Tóxicos en Levadura (ATL) family of membrane localized E3 ligases consists of over 90 members with vastly varied functions (Guzmán, 2012). All ATLs share a set of protein domains, including the Really Interesting New Gene (RING) E3 Ligase domain, a hydrophobic region near the N-terminus (apparent transmembrane domains), and a C-terminal region with unknown function, proposed to play a role in substrate recognition (Serrano et al., 2006; Guzmán, 2012). ATLs have been shown to regulate a range of responses, including cold and drought stress (ATL78) (Kim and Kim, 2013), programmed cell death (ATL55) (Lin et al., 2008), the photoperiod response (ATL62) (Morris and Jackson, 2010; Morris et al., 2010), and the C/N nutrient response (ATL31) (Sato et al., 2009). In addition, several ATLs have been loosely implicated in defense responses based on increases in transcript accumulation after PAMP treatment (Salinas-Mondragón et al., 1999; Serrano and Guzmán, 2004; Berrocal-Lobo et al., 2010). ATLs have been shown to play roles in immunity in other species, such as rice and
tomato, as well (Takai et al., 2001; Durrant et al., 2000; Hondo et al., 2007). However, no genetic mutants lacking a member of the ATL family have been reported that result in altered defense responses.

Previous work in our lab found that ATL6 is rapidly phosphorylated in response to flg22, potentially implicating it in a PTI-associated defense response (Nühse et al., 2007). Due to its rapid phosphorylation after elicitation and its localization to the plasma membrane, ATL6 is a candidate for modulating the PM proteome during a PTI response. Previous reports showed that ATL6 transcripts accumulate after flg22 treatment, and ATL6 overexpression confers a slight increase in resistance to the plant (Maekawa et al., 2012). However, no known or proposed mechanism has been postulated for this result, and no defense phenotypes have been reported for atl6 mutants.

Here, I use knockout mutants to demonstrate an important role for ATL6 in PTI. When pretreated with flg22 or infected with a compromised strain of the pathogen (Pst DC3000ΔavrPto/avrPtoB), atl6 knockout plants show increased susceptibility compared to wild type plants, indicating ATL6 is required for a robust, PTI-related defense. Interestingly, unlike all other E3 ligases associated with PTI, all early molecular responses tested were unaltered in atl6 mutants, suggesting that ATL6 functions downstream of the initial perception and subsequent immediate signaling. The atl6 mutants fail to restrict bacterial effector delivery after PAMP treatment, indicating that ATL6 may play a role in regulating the extracellular accumulation of bioactive metabolites during PTI. Because
effector delivery restriction is also seen in the mkp1 mutant, I used an atl6 mkp1 double mutant to investigate a possible genetic interaction between the two proteins. The atl6 mutation suppressed the enhanced resistance of mkp1 mutants, indicating that ATL6 functions downstream of the MKP1-regulated pathway(s) to regulate PTI defense. Together, these results establish ATL6 as a novel component of PTI and place the function of this protein downstream of the MKP1-regulated defense pathway.

Results

**ATL6 and its two phosphorylation sites are conserved among plant species**

ATL6 is a RING-type plasma membrane-localized E3 ubiquitin ligase in *Arabidopsis thaliana* containing a signal peptide, a transmembrane domain, a Really Interesting New Gene (RING) E3 ligase domain, and a C-terminal domain (Figure 2.1A). A previous phosphoproteomics study found that upon flg22 elicitation, phosphorylation of ATL6 increased at two serine residues (Ser-278 and Ser-357) in the C-terminal region (Figure 2.1A) within minutes after elicitation (Nühse et al., 2007) If phosphorylation of these sites is important for defense-related regulation of ATL6, implicating ATL6 as playing a role in defense responses, it would be expected that these phosphorylation sites would be highly conserved throughout evolution. To investigate this possibility, Basic Local Alignment Search Tool (BLAST) was used to identify apparent ATL6 orthologs in other species; and the alignment of the sequences surrounding both Arabidopsis phosphorylation sites
showed that the sequences were highly conserved across the plant kingdom, even within monocots (Figure 2.1B). The minimal R$_3$-X-X-S phosphorylation motif of both sites indicates that ATL6 may be a substrate of calcium-dependent protein kinases (CDPKs), SNF-related serine/threonine-protein kinases (SnRKs), and/or the CBL-interacting protein kinases (CIPKs), which will be discussed in more detail in a later chapter. Because we identified ATL6 as becoming rapidly phosphorylated during pattern-triggered immunity (PTI) elicitation and because the phosphorylation sites we identified were highly conserved across evolution, indicating that the proteins are regulated during PTI, we decided to pursue the isolation of genetic mutants to investigate the possible role(s) of ATL6 during plant immune responses.
Figure 2.1: ATL6 is a PM-localized E3 Ubiquitin Ligase with two conserved phosphorylation sites.

(A) Model showing domain architecture of ATL6, including signal peptide (SP), transmembrane domain (TM), a RING-type E3 domain (RING) and a C-terminal domain (C-Terminal). Arrows denote conserved phosphorylation sites. (B) Sequence alignment showing conserved phosphorylation sites across several plant species. Gray shading shows R-X-X-S motif.
**Isolation of two atl6 T-DNA knockout mutants**

To study the potential importance of ATL6 in PTI, two independent T-DNA knockout lines were isolated and denoted *atl6-1* and *atl6-2* (SALK_083652 and SALK_134489, respectively). As ATL6 is an intronless gene, both T-DNA insertions interrupt the single exon (Figure 2.2A). To confirm both mutant lines were homozygous knockouts, PCR genotyping was performed using the primers from Table 2.1 (Figure 2.2B). In both alleles, there were no noticeable morphological differences compared with Col-0 (WT) (Figure 2.2C). Confirmation that the insertion of the T-DNA into the exon disrupted the proper accumulation of ATL6 was obtained by two, independent methods. RT-PCR using the primers from Table 2.2 showed that ATL6 transcript was significantly reduced in the mutants (Figure 2.3A) and immunoblot analysis using an αATL6 antibody showed the absence of a protein band of the expected size in mutants (Figure 2.3B). These results demonstrated that we had isolated homozygous insertional mutants that decreased the levels of ATL6 to undetectable levels.
Figure 2.2: Isolation of two independent *atl6* T-DNA insertion lines.

(A) Model showing ATL6 gene structure as well as positions of T-DNA insertions. (B) PCR genotyping of two independent *atl6* mutants (*atl6*-1, *atl6*-2) using primers shown in Table 2.1. (C) Photographs of 5-week-old Col-0, *atl6*-1, and *atl6*-2 plants. Genotyping was done and photographs were taken by Lingyan Jiang.
Figure 2.3: Confirmation of two independent *atl6* T-DNA insertion lines.

(A) RT-PCR of Col-0, *atl6*-1, and *atl6*-2 plants using the RT-PCR primers shown in Table 2.2. (B) Immunoblot analysis of Col-0, *atl6*-1, and *atl6*-2 plants using an αATL6 antibody with αCNX1/2 as a loading control. Asterisk (*) indicates nonspecific band.
ATL6 is a positive regulator of PTI-associated resistance

To determine if ATL6 plays a role in resistance, I first performed pathogen infection assays by surface inoculation of 12-day-old plants using virulent Pseudomonas syringae pv tomato DC3000 (Pst DC3000). Three days post-infection (dpi), bacterial levels were quantified, and no significant difference was observed between Col-0, atl6-1, and atl6-2 (Figure 2.4A left). Pst DC3000 is very effective in overcoming PTI resistance in Arabidopsis because of its ability to inject effector proteins; so, I also performed pathogen assays using a partially compromised mutant lacking two of the predominant PTI-suppressing effectors (Pst DC3000ΔavrPto/ΔavrPtoB). This DC3000 mutant has been demonstrated to be effective in detecting plant mutations altering PTI-mediated defenses because it does not fully suppress PTI but still retains its other effectors, allowing it to grow on Arabidopsis if the plant’s PTI is not fully functional (Lin and Martin, 2007). Growth of Pst DC3000ΔavrPto/avrPtoB was significantly reduced in Col-0 plants, as compared to the fully virulent strain, but it grew to levels seen in the fully virulent pathogen in both alleles of the atl6 mutants, indicating failure in these mutants to properly manifest resistance (Figure 2.4A right).

As an independent approach to assess PTI, plants were surface treated with 1 µM flg22 for 8 hours before surface infection with virulent Pst DC3000. As expected, Col-0 plants pre-treated with flg22 showed a strong decrease in bacterial growth, indicating that a robust PTI-associated defense response could suppress growth of the virulent pathogen. However, both atl6-1 and atl6-2 showed no
significant reduction in bacterial growth after pre-treatment with flg22 as compared to naïve plants (Figure 2.4B), indicating that plants lacking ATL6 are compromised in their ability to manifest a PTI-associated defense response.

I also performed pathogen assays with a highly compromised mutant of Pst DC3000 without a functional type-III secretion system (T3SS) \([\text{Pst DC3000} \text{(hrcC)}]\). Infection with \(\text{Pst DC3000 (hrcC)}\) consistently resulted in slightly higher bacterial growth in the \(\text{atl6}\) mutants as compared to Col-0 (Figure 2.4C). Although the hrcC-mutant is sometimes used to investigate PTI-related defenses because of its lack of effectors, the mutant grows poorly when using a passive (i.e., not infiltrating) infection, hence the smaller differences in bacterial levels compared to the previous experiments.

To determine if ATL6 is required to manifest a full PTI response in older, soil-grown plants, adult plants (4-5-weeks old) were spray inoculated with \(\text{Pst DC3000}\Delta\text{avrPto/avrPtoB}\); and colony forming units (cfus) were quantified 3 days post-infection (dpi). Similar to what we observed in seedlings, both \(\text{atl6-1}\) and \(\text{atl6-2}\) plants showed significantly increased pathogen growth compared with Col-0, indicating \(\text{atl6}\) plants are compromised in their ability to manifest PTI-induced resistance at multiple stages of plant development (Figure 2.5). Taken together, these results demonstrate that ATL6 is a positive regulator of PTI-associated defense responses in plants.
Figure 2.4: *atl6* mutants are not able to mount a PTI-associated defense response

(A and C) 12-day-old seedlings were immersed in $2 \times 10^7$ cfu mL$^{-1}$ solution of *Pst* DC3000 and *Pst* DC3000ΔAvrPto/AvrPtoB (A) or *Pst* DC3000 (hrcC$^-$) (C) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ (2 seedlings per sample) before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Asterisks indicate significant differences from Col-0 using an ANOVA (A) or a t-test compared with Col-0, (C) * P<0.05, **** P<0.0001. (B) 12-day old seedlings were pre-treated with 1 µM flg22 followed by infection with a $2 \times 10^7$ cfu mL$^{-1}$ solution of *Pst* DC3000. Cfus were calculated as described above. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Asterisks indicate significant differences from Col-0 using a t-test, **** P<0.0001.
**Figure 2.5: Adults atl6 mutants are susceptible to a compromised strain of *Pst* DC3000.**

4-5-week-old plants were spray inoculated with a $1 \times 10^8$ cfu mL$^{-1}$ solution of *Pst* DC3000Δ*avrPto/avrPtoB* with 0.04% Silwet. 3 dpi, leaf punches (0.2 cm$^2$) were taken and ground in 10 mM MgCl$_2$ (2 leaf punches per sample) and cfu quantification was carried out as described previously. Values are means ± SEM (n=18) pooled from 3 individual experiments. Asterisks indicate a significant difference using a one-way ANOVA, **P<0.01, ***P<0.001.
**PRR levels and signaling are not altered in *atl6* mutants**

Due to *atl6* plants’ lack of ability to manifest a PTI-associated defense response, I wanted to investigate which portions of PTI responses may be altered, thereby providing information about where ATL6 may function in this process. One possibility was that the absence of ATL6 may result in a defect in pattern recognition receptor (PRR) accumulation or signaling. PRRs localize to the plasma membrane and perceive the presence of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) to initiate downstream signaling. If PRRs do not accumulate to necessary levels, the plant will be deficient in its ability to perceive PAMPs and, therefore, its ability to manifest a full PTI response. To directly examine Flagellin-sensing 2 (FLS2) abundance, an αFLS2 antibody was used to probe blots with proteins from both whole seedling tissue and enriched microsomal fractions of Col-0 and *atl6*-1 plants (Figure 2.6A). No difference in FLS2 levels was found in either case, indicating that *atl6*-1 plants accumulate FLS2 normally. Previous work has shown that endocytosis and subsequent degradation of FLS2 after elicitation is associated with proper PTI responses (Smith *et al.*, 2014). To evaluate whether ATL6 may be required in this process, Col-0 and *atl6*-1 plants were treated for one hour with 10 µM flg22, and protein levels were analyzed by immunoblot with the αFLS2 antibody (Figure 2.6B). In both Col-0 and *atl6*-1 plants, FLS2 levels were considerably reduced after flg22 treatment compared to unelicited plants, indicating removal of the receptor. Together these data suggest
that \textit{atl6-1} plants are able to effectively accumulate FLS2. Therefore, ATL6 does not
appear to be involved in regulating the levels of FLS2.

I also wanted to investigate if an independent PRR, the EF-Tu receptor
(EFR), accumulated and/or was trafficked properly in \textit{atl6} plants. Because there is
not an αEFR antibody available, MAPK activation by phosphorylation (a response
that occurs rapidly after PRR activation) can be used as a proxy for elf18 perception
and signaling. After Col-0 plants were elicited with 10 μM elf18, the αpTEpY
antibody detected more of phosphorylated MPK3 and MPK6 (Figure 2.7; -elf18 1\textsuperscript{st}
elicitation, +elf18, 2\textsuperscript{nd} elicitation), indicating that elf18 treatment resulted in an
increase of MAPK activation. As MAPK activation in the mutants is
indistinguishable from that in the wild type plants, ATL6 does not appear to play
a role in MAPK phosphorylation. However, if plants were pre-treated with elf18
one hour prior to the second elicitation, treatment again with elf18 did not result
in MAPK phosphorylation in either Col-0 or the \textit{atl6} mutants (Figure 2.7; +elf18 1\textsuperscript{st}
elicitation, +elf18 2\textsuperscript{nd} elicitation). This result could occur if (a) MAPK activation is
directly affected by the first elicitation to prevent a second elicitation or (b) EFR
was no longer present at the plasma membrane in the proper quantity to
effectively perceive the PAMP and initiate downstream signaling. To distinguish
between these possibilities, a second elicitation with pep1, another elicitor
perceived by PEPR1, was used to test for MAPK activation. In both Col-0 and the
\textit{atl6} mutants, pretreatment with elf18 did not prevent MAPK activation by pep1
(Figure 2.7; +elf18 1\textsuperscript{st} elicitation, +pep1 2\textsuperscript{nd} elicitation), demonstrating that the
MAPKs were still capable of being activated through phosphorylation. Therefore, the lack of activation observed in the plants treated twice with elf18 is due to an alteration in elf signaling and not the ability of the plant to activate the MAPKs. These results are consistent with the model that after the first elf18 treatment, EFR is being endocytosed and/or degraded in a ligand-induced manner such that it is not present to perceive the second elicitation (Mbengue et al., 2016). All together, these results indicate that atl6 plants are not altered in their ability to perceive elf18 or in the PAMP-induced activation of MAPKs.
Figure 2.6: FLS2 levels as well as ligand-induced FLS2 degradation are unaltered in *atl6-1* plants.

(A) Immunoblot analysis of microsomal (left) and total (right) protein fractions from Col-0 and *atl6-1* seedlings were probed with αFLS2 antibodies. Microsomal enrichment was confirmed using an αAHA antibody. CBB was used as a loading control. Each sample was 60 seedlings pooled. Results are representative of three independent experiments. Naïve FLS2 level experiment was carried out by Lingyan Jiang. (B) Leaf punches (0.2 cm$^2$) were taken from 4-5-week-old plants and sliced into 5 leaf strips (2 discs; 10 strips per sample). Samples were elicited or not with 10 μM flg22 for one hour. Total protein samples were then probed with an αFLS2 antibody using αMPK6 as a control. Results are representative of two independent experiments.
Figure 2.7: De-Sensitization of PRR signaling is not altered in *atl6* mutants.

12-day-old seedlings were elicited for one hour using 10 µM elf18. Plants were then subsequently elicited with either another treatment of 10 µM elf18 or 1 µM pep1 to analyze de-sensitization of PRR signaling. A αpTEpY antibody was used to probe for MPK6/MPK3 activation with αMPK6 used as a loading control. Results are representative of three individual experiments.
**ATL6 is not required for a variety of PTI-associated molecular responses**

To further explore where ATL6 might function in PTI, I examined several PTI responses that are markers for distinct signaling pathways. Reactive Oxygen Species (ROS) are produced in an early Ca\(^{2+}\)-dependent response and accumulate rapidly after elicitation with flg22. To determine if ATL6 is involved in early Ca\(^{2+}\) responses, ROS was quantified in Col-0, *atl6-1*, and *atl6-2* plants after treatment with 1 µM flg22. As expected, treatment with flg22 resulted in an increase in ROS in Col-0 (Figure 2.8A), and there was no significant difference in either total ROS produced (Figure 2.8A) or peak ROS (Figure 2.8B) in *atl6-1* or *atl6-2* plants compared to wild type. These results indicate that ATL6 does not play a role in regulating flg22-induced ROS production. I also examined total ROS production after treatment with another PAMP, 1 µM elf18 (Figure 2.8C), and the DAMP 1 µM pep1 (Figure 2.9D). In all cases, treatment with elicitors led to a transient accumulation of ROS; but there was no difference in ROS production between Col-0 plants and the *atl6* mutants, suggesting ATL6 does not play a role in ROS production, regardless of which elicitor is used. Together, these data suggest that ATL6 does not play a role in elicitor-dependent ROS accumulation.

After PAMP elicitation, different defense-related transcripts accumulate as a result of the activation of several independent pathways. To probe these changes in transcript abundance, I elicited Col-0, *atl6-1*, and *atl6-2* plants with 1 µM flg22 for various amounts of time. qRT-PCR was then used to quantify transcripts from several pathways, including Ca\(^{2+}\)- (NHL10), MAPK- (FRK1), and SA- (PRI)
dependent pathways (primers listed in Table 2.2) (Boudsocq et al., 2010; Tsuda et al., 2008). In all cases, the flg22-induced accumulation of transcripts did not show any differences between genotypes (Figure 2.9). Therefore, ATL6 does not regulate accumulation of defense-related transcripts across any of the examined pathways.

During a defense response, a plant attempts to redirect energy and nutrients towards defending itself against a pathogen at the expense of growth and development. These responses result in a PAMP-induced inhibition of plant growth. To determine if ATL6 plays a role in PTI-associated growth inhibition, six-day-old Col-0 and atl6-1 plants were elicited with increasing concentrations of flg22 for twelve days, and fresh weight was measured (Figure 2.10). As expected, Col-0 plant growth was increasingly restricted at higher flg22 concentrations, indicating successful growth inhibition. atl6-1 plants also displayed increasing growth inhibition with no significant difference from Col-0 plants, indicating ATL6 does not play a role in regulating growth inhibition. Taken together, these results demonstrate a potentially novel role for ATL6 in PTI, as no canonical responses were altered despite atl6 mutants lacking the ability to mount a robust PTI-associated defense response.
Figure 2.8: ROS accumulation is unaltered in *atl6* mutants using a variety of PAMPs/DAMPs.

(A-D) Leaf discs (0.2 cm²) were taken from 4-5-week-old plants and bisected before being placed in wells of a white 96-well plate. After equilibrating in sterile H₂O overnight, solution was exchanged for luminol dissolved in 0.2 M KOH with 20 µg mL⁻¹ horseradish peroxidase and either 1 µM flg22 (A and B), elf18 (C), or pep1 (D). Relative Light Units (RLUs) were measured to calculate relative total ROS (A, C, and D) or peak ROS (B). Values are means ± SEM (n=64) pooled from 3 independent experiments. No significant differences were observed using a t-test compared to Col-0, P<0.05.
Figure 2.9: Accumulation of several defense related transcripts is unaltered in *atl6* mutants.

12-day-old seedlings were elicited with 1 µM flg22 for 30 minutes (A), 3 hours (B), or 24 hours (C). Triplicate samples were frozen, and RNA was isolated with 6 seedlings per sample. cDNA was synthesized and qRT-PCR was carried out using technical duplicates to quantify *NHL10* (A), *FRK1* (B), and *PR1* (C), transcripts using At2g28390 as a reference gene. Primer sequences are found in Table 2.3. Values are mean ± SEM (n=3) representing three independent experiments. No significant differences were observed within single time points using a t-test compared to Col-0, P<0.05.
Figure 2.10: flg22-induced growth inhibition is unaltered in \textit{atl6-1} plants.

6-day-old plants were placed in 0.5x liquid MS media in 24-well plates (one seedling per well) along with varying concentrations of flg22. After 12 days of elicitation, fresh weight was measured for each individual seedling. Values are means ± SEM (n=36) pooled from 3 independent experiments. No significant differences were calculated among individual flg22 concentrations using a t-test, P<0.05.
**ATL6 plays an important role in PTI-associated effector delivery restriction**

During an infection, one of the primary strategies of *Pseudomonas syringae* to colonize the host is to activate its type-III secretion system (T3SS) to inject effector proteins into the plant cell and inhibit defense responses. Several effectors, including AvrPto and AvrPtoB, actively disrupt PTI responses in the plant by targeting PTI machinery. Previous work has shown that after PAMP perception, the plant attempts to disrupt this strategy by actively restricting effector delivery (Crabill *et al.*, 2010; Oh *et al.*, 2010). To determine if ATL6 may be involved in this aspect of plant defense, an effector delivery assay was used to determine if PTI-induced restriction of effector delivery was altered in *atl6-1* plants. 12-day-old seedlings were elicited with 1 µM flg22 for eight hours before subsequent infection with *Pst* DC3000 expressing the effector protein AvrPto fused to adenylyl cyclase (*Pst* DC3000 (AvrPto-CyA)) (Schechter *et al.*, 2006). After the effector is translocated into the plant cell, it produces cAMP in the presence of eukaryotic calmodulin. cAMP is only produced after effectors have been injected into the plant cell, allowing for effector delivery to be quantified in an ELISA using cAMP levels as a proxy for delivery. As expected, pretreating Col-0 plants with flg22 resulted in a significant decrease in effector delivery (Figure 2.11). This result indicates that during a PTI-associated defense response, wild type plants are able to restrict the delivery of effector proteins into the plant cell. In contrast, *atl6-1* plants displayed no significant difference between naïve and flg22-elicited plants, indicating that ATL6 is required for the proper suppression of bacterial effector
delivery. In summary, a plausible model from all the data is that ATL6 functions downstream in the PTI pathway near the site of action for controlling the restriction of bacterial effector delivery. This interpretation would explain why all the PTI responses that are normally assayed were normal in \textit{atl6} mutants. Thus, ATL6 appears to be a novel regulator of PTI in that resistance fails to fully manifest in knockout mutants even though all the canonical responses tested occur normally. These results also indicate that restriction of effector delivery is a key aspect of PTI.
Figure 2.11: flg22-induced effector delivery restriction is suppressed in atl6-1 plants.

12-day-old seedlings were elicited with flg22 for 24 hours before infecting with $2 \times 10^7$ cfu Pst DC3000 (AvrPto-CyA) for a further 24 hours. Samples of 2-6 seedlings were ground in 0.1 M HCl and debris was pelleted. cAMP in supernatant was measured using a direct cAMP ELISA kit. Values are means ± SEM (n=20) pooled from 5 independent experiments. Letter groups indicate a statistical difference determined using a two-way ANOVA, P<0.05.
**ATL6 is required for the resistance observed in *mkp1* plants**

During a PTI response, a primary objective of the plant is to restrict pathogen effector delivery into the plant cell. Previous work in the lab has demonstrated that *mkp1* plants mimic PTI-induced plants in their ability to restrict effector translocation into the plant cell (Anderson *et al.*, 2014). This restriction of effector delivery was due to a decrease in extracellular accumulation of a subset of bioactive organic acids required by DC3000 to activate its T3SS. A likely explanation for this decrease in extracellular metabolites is altered regulation and/or abundance of transporters at the plasma membrane in the *mkp1* mutant and after PTI elicitation. Because ATL6 is a PM-localized E3 Ligase, is a positive regulator of PTI-associated resistance, and is required for restricting effector delivery, I hypothesized a potential genetic link between ATL6 and MKP1. To study this relationship, a double knockout mutant was generated by crossing the *atl6-1* mutant with the *mkp1* mutant (Figure 2.12); and PCR genotyping was used to confirm a homozygous double knockout (Primers used described in Table 2.1). RT-PCR was used to confirm that both *ATL6* and *MKP1* transcripts were undetectable (Figure 2.3A).

To test for potential genetic interactions between ATL6 and MKP1, a pathogen infection assay was performed using 12-day-old Col-0, *atl6-1*, *mkp1*, and *atl6-1 mkp1* seedlings that were surface inoculated with *Pst* DC3000. As expected, *atl6-1* plants showed the same level of growth of the virulent pathogen as seen in
Col-0, while *mkp1* plants showed significantly lower bacterial levels, indicating enhanced resistance. Interestingly, in the *atl6-1 mkp1* double mutant, bacteria grew to the same levels as Col-0, indicating that the enhanced resistance in *mkp1* plants is suppressed in the *atl6-1 mkp1* double mutants. These results indicate that the resistance in *mkp1* plants is at least partially dependent upon ATL6 (Figure 2.13A). I also performed a surface infection assay on soil-grown adult plants. Like with the seedling assays, *atl6-1* infection levels closely matched those of Col-0, while *mkp1* plants showed significantly decreased bacterial levels, showing that *mkp1* resistance is maintained in adult plants. In *atl6-1 mkp1* double mutant plants, however, bacteria grew to the same levels as in wild type plants, demonstrating suppression of resistance in *mkp1* mutants if the plants also lacked ATL6 (Figure 2.13B). Taken together, these data indicate that ATL6 functions genetically downstream of MKP1.
Figure 2.12: Generation of a double knockout mutant of *atl6-1 mkp1*.

PCR genotyping of *atl6-1 mkp1* double mutant leaf tissue generated from a cross between *atl6-1* and *mkp1* plants using primers found in Table 2.1. Tissue generation and genotyping was done by Lingyan Jiang.
Figure 2.13: Resistance to *Pst* DC3000 observed in *mkp1* plants is dependent on ATL6.

(A) 12-day-old seedlings were infected with $2 \times 10^7$ cfu *Pst* DC3000. 3 dpi, cfus were calculated as described in Figure 2.3. Values are means ± SEM (n=18) pooled from three individual experiments. Asterisks indicate significant difference calculated from Col-0 using a t-test, **** $P<0.0001$. (B) 4-5-week-old plants were spray inoculated with a $1 \times 10^8$ cfu mL$^{-1}$ solution of *Pst* DC3000 with 0.04% Silwet. 3 dpi, leaf punches (0.2 cm$^2$) were taken and ground in 10 mM MgCl$_2$ (2 leaf punches per sample) and cfu quantification was carried out as described previously. Values are means ± SEM (n=6) from 1 experiment. Asterisks indicate a significant difference from Col-0 using a t-test, *** $P<0.01$. 

Col-0  atl6-1  mkp1  atl6-1 mkp1

Col-0  atl6-1  mkp1  atl6-1 mkp1
ATL6 does not play a role in a number of other mkp1-associated molecular phenotypes

In addition to its role in pathogen resistance, MKP1 has previously been shown to be a negative regulator of several other PTI-associated phenotypes, including ROS production and defense-related transcript accumulation. To better understand the relationship between ATL6 and MKP1, I investigated if some of these alterations were also suppressed in the atl6-1 mkp1 double mutant.

mkp1 plants have been previously shown to hyperaccumulate ROS after PAMP elicitation (Anderson et al., 2011). To determine if ATL6 plays a role in this response, a flg22-induced ROS production assay was carried out as described above on the atl6-1 mkp1 double mutant. Consistent with previous results, ROS hyper-accumulated in mkp1 mutant plants compared to Col-0. However, this hyper-accumulation was not suppressed in the atl6-1 mkp1 double mutants, indicating this phenotype is independent of ATL6 (Figure 2.14).

qRT-PCR was then used to assess the accumulation of a defense-related transcript. In a previous study, mkp1 plants accumulated higher levels of a subset of defense-related transcripts after elicitation with the PAMP elf18 (Anderson et al., 2011). Here, I show that mkp1 plants also hyperaccumulate NHL10, a marker gene for the CDPK pathway, in response to treatment with 1 µM flg22. NHL10 accumulation did not show any reduction in the atl6-1 mkp1 double mutant, indicating that this response is also independent of ATL6 (Figure 2.15). Together these results indicate that ATL6 does not play a role in other previously described
mkp1-associated early molecular phenotypes. This suggests that ATL6 is functioning downstream of MKP1, but in a later or independent pathway in resistance compared to other molecular responses.
Figure 2.14: Hyper-accumulation of ROS observed in \textit{mkp1} plants is independent of ATL6.

Leaf discs (0.2 cm\(^2\)) were taken from 4-5-week-old plants and bisected. Half leaf discs were then elicited with 1 \(\mu\)M flg22 in the presence of luminol dissolved in 0.2 M KOH and 20 \(\mu\)g mL\(^{-1}\) horseradish peroxidase. RLU\(\text{s}\) were measured and relative total ROS was calculated. Values are means \(\pm\) SEM (n=72) pooled from 4 individual experiments. Letter groups represent significant differences calculated from a one-way ANOVA, \(P<0.05\).
Figure 2.15: Hyper-accumulation of defense-related transcripts in *mkp1* plants is independent of ATL6

12-day-old seedlings were elicited with 1 µM flg22 for 30 minutes. RNA was isolated from triplicate samples (6 seedlings per sample) and cDNA was synthesized. qRT-PCR was used with technical duplicates to quantify NHL10 transcripts. *At2g28390* was used as a reference gene and samples were normalized to unelicited Col-0 plants. Sequences for primers used are found in Table 2.3. Values are means ± SEM (n=3) representative of three independent experiments. Letter groups representative significant differences using a one-way ANOVA, P<0.001.
Preliminary evidence suggests ATL6 may be required for increased growth inhibition in mkp1 plants

In the Ws ecotype, mkp1 knockout plants show much greater growth inhibition compared to wild-type plants after elicitation with elf18 (Anderson et al., 2011). To determine if ATL6 plays a role in this mkp1-dependent growth inhibition, the assay was carried out in the Col-0 background with the single and double mutants. Because mkp1 plants in the Col-0 background begin to develop growth inhibition in a SNC1-dependent manner (Bartels et al., 2009), untreated mkp1 plants are significantly smaller than wild-type plants (Fig. 2-17; -flg22). Interestingly, the atl6-1 mkp1 double mutant partially suppresses this phenotype in the absence of elicitor. After treating with flg22 (100 nM and 10 µM), the increased growth inhibition observed in mkp1 plants is completely reverted to Col-0 levels in the atl6-1 mkp1 double mutant (Figure 2.16). This result suggests that ATL6 plays a role in the increased growth inhibition observed in mkp1 (Col-0), both before and after PAMP elicitation. However, this is slightly complicated by the fact that, in contrast to Figure 2.10, atl6-1 plants were slightly larger than Col-0 after 100 nM flg22 treatment. This potentially suggests an addictive effect, resulting in the atl6-1 mkp1 plants being larger than the mkp1 mutants.
Figure 2.16: Enhanced flg22-induced growth inhibition observed in *mkp1* plants is suppressed in *atl6-1 mkp1* double mutant plants.

6-day-old seedlings were placed in 0.5x liquid MS in a 24-well plate with varying amounts of flg22. After 12 days of elicitation, plants were weighed individually. Values are means ± SEM (n=42) pooled from three individual experiments. Letter groups indicate significant differences within each flg22 concentration using a two-way ANOVA, *P*<0.0001 (-flg22), *P*<0.01 (100 nM flg22), *P*<0.05 (10 µM flg22).
Discussion

**ATL6 functions as a novel regulator of PTI**

We had previously found that ATL6 is phosphorylated rapidly after flg22 treatment (Nühse et al., 2007), and PAMP elicitation induces ATL6 mRNA transcript accumulation (Maekawa et al., 2012). However, other than this loosely correlative molecular data, no defense-related phenotypes have been reported for ATL6 using genetic mutants; and no mechanisms have been hypothesized. In this work, two knockout alleles, atl6-1 and atl6-2, were utilized to examine the role of ATL6 in PTI. Although atl6 mutants are not more susceptible to fully virulent Pst DC3000, plants lacking ATL6 are compromised in their ability to properly manifest a PTI-associated defense response. atl6 mutants do not develop PTI-mediated restriction of pathogen growth after PAMP elicitation of the plants, and they are not able to fully restrict the growth of strains of Pst DC3000 that are compromised in their ability to suppress PTI (Figure 2.4). These results provide the first genetic evidence that ATL6 is required for a full PTI response.

A potential hypothesis for where ATL6 may function is that it is involved in the perception of PAMPs in early PTI-associated responses. Several other E3 Ligases (e.g., PUB12/13/22/23/24 and RHA3A/B) have been implicated in targeting receptor complex components for endocytosis or degradation, resulting in aberrant PTI responses in mutants lacking these proteins (Lu et al., 2011; Trujillo et al., 2008; Stegmann et al., 2012; Ma et al., 2020). In addition, PIRE has been shown to target the NADPH oxidase RBOHD for degradation, resulting in increased ROS
production in pire mutants (Lee et al., 2020). In all these previously described mutants, early molecular phenotypes, such as FLS2 accumulation and recycling, ROS production, or defense-related transcript accumulation, are altered. Therefore, their mis-regulation of PTI appears to be explained largely by the fact that by interfering with early signal propagation, most of the subsequent PTI-related responses are altered. In contrast, although atl6 plants clearly fail to mount an effective PTI response, receptor levels and activity remain unchanged from wild type plants; and all of the canonical molecular PTI-associated phenotypes examined were unaltered. These results suggest that unlike all previously described E3 ligases, ATL6 is playing a role either further downstream in PTI or in a previously undefined pathway.

**ATL6 functions with MKP1 during a defense response to restrict effector delivery**

Previous work showed that the enhanced resistance in mkp1 mutant plants occurs by restricting the accumulation of extracellular, T3SS-inducing metabolites. This decrease in extracellular metabolites results in large reductions in the bacterial pathogen’s ability to express effector proteins and subsequently inject them into the plant cell, and the inability of the pathogen to deliver its effectors results in increased resistance against the pathogen (Anderson et al., 2014). In addition, exogenous replacement of these metabolites suppressed the enhanced resistance in both mkp1 mutants and in PAMP-pretreated plants, demonstrating
the importance of the plant’s ability to control the extracellular levels of these T3SS-inducing signals for a proper PTI response (Anderson et al., 2014). One hypothesis for how this decrease in bioactive metabolite accumulation could occur is that transporters secreting these metabolites at the plasma membrane have either their activity or their abundance decreased during a PTI response. Due to its localization to the PM and its role as an E3 ligase functioning later in PTI responses, ATL6 is a potential candidate to be involved in regulating these transporters. Because mkp1 is the only mutant known to alter the extracellular accumulation of these metabolites, we generated a double knockout, atl6-1 mkp1, to investigate a possible genetic link between ATL6 and MKP1. While mkp1 plants are more resistant to infection, the double mutants suppressed this enhanced resistance, indicating that resistance in mkp1 plants is dependent on ATL6, placing ATL6 downstream in the pathway mis-regulated in mkp1 mutants (Figure 2.13). Because the only other protein known to be required for resistance in mkp1 plants is the direct substrate of MKP1, MPK6 (Anderson et al., 2011), ATL6 is likely to function downstream of MPK6 signaling during PTI responses. This conclusion would be consistent with the previously stated observation that the other early PTI responses examined in atl6 mutants were not altered, as these would be either upstream or independent of this placement in PTI signaling.

mkp1 plants as well as PAMP-elicited plants both restrict effector delivery. Since ATL6 is required for mkp1-associated and PAMP-induced resistance, I hypothesize that ATL6 also functions in restricting effector delivery. After PAMP
pretreatment, wild-type plants mount a PTI-associated defense response to restrict bacterial effector delivery into the plant cell as well as bacterial growth (Crabill et al., 2010; Oh et al., 2010). \textit{atl6} knockout plants, however, do not show inhibition of \textit{flg22}-induced effector delivery. This, as well as the fact that it is required for \textit{mkp1}-associated resistance, suggests that ATL6 positively regulates PTI by controlling the restriction of T3SS-inducing metabolites. A necessary follow up experiment will be to perform effector delivery assays in the \textit{atl6-1 mkp1} double mutant, although preliminary data suggests that ATL6 is, in fact, required for the restriction of effector delivery in \textit{mkp1} plants.

In addition to restricting effector delivery, \textit{mkp1} plants have several other defense-related phenotypes, such as increased ROS production and hyperaccumulation of defense-related transcripts after PAMP treatment (Anderson et al., 2011). Interestingly, these phenotypes are independent of ATL6, as they are not suppressed in \textit{atl6-1 mkp1} double mutants. These results suggest that ATL6 plays a role more specifically in restricting effector delivery during a PTI response, which is downstream of one branch of the signaling pathways regulated by MKP1. A working model is that after the initial perception of a PAMP, the MAPKs are activated, with one of the indirect downstream targets being the phosphorylation and activation of ATL6, which then either inactivates or removes currently undefined metabolite transporters in the plasma membrane (Figure 2.17). This model would provide an explanation for why ATL6 is rapidly phosphorylated during a PAMP response and why other molecular phenotypes
are not altered in atl6 mutants. In mkp1 knockout mutants, MPK6 would be hyperactivated during a PTI-associated response, leading to more ATL6 activity, resulting in more effective removal of transporters from the PM, explaining the restricted effector delivery and increased resistance in mkp1 plants. Based on the phosphorylation motifs found in PAMP-regulated ATL6 phosphorylation sites, ATL6 cannot be a direct substrate of MPK6 as all MAPKs are proline-directed kinases; and there is not a proline in the +1 position of either of the phosphorylation sites (Taj et al., 2010). Therefore, there must be at least one other, currently unknown, kinase between MPK6 and ATL6. The possible identity of this new kinase will be discussed in a subsequent chapter of this thesis.

Based on the conclusions summarized above, I have discovered that ATL6 is a novel, positive regulator of PTI. Its apparent role in restricting the accumulation of bioactive, T3SS-inducing metabolites fills in an important, missing piece in our understanding of how PAMP treatment results in enhanced resistance during PTI. In addition, this protein also provides a new entry point both to identify the specific transporters involved and to identify the missing kinase that links MPK6 to ATL6, thus revealing another novel component of the signaling pathway(s) leading to PTI resistance.
Figure 2.17: Hypothesized role for ATL6 in a PTI-associated defense response.

Cartoon model showing a PTI-associated defense response in (A) Col-0, (B) atl6-1, (C) mkp1, and (D) atl6-1 mkp1 plants. (A) ATL6 functions downstream of MPK6 to regulate transporter accumulation during PTI. (B) Deletion of ATL6 results in an increase of metabolite transporters at the PM, leading to more extracellular metabolites and, therefore, increased susceptibility. (C) Deletion of MKP1 leads to hyper-activation of MPK6, resulting in hyper-activity of ATL6, leading to more metabolites being targeted for removal from the PM. (D) Deletion of both ATL6 and MKP1 leads to more accumulation of transporters at the PM, resulting in increased extracellular metabolite accumulation, and therefore increased susceptibility.
Materials and Methods

Plant Material and Growth Conditions

Arabidopsis seeds were sterilized using a 10% bleach and 0.05% Tween-20 solution for 15 minutes at room temperature while spinning before being thoroughly washed with sterile H$_2$O. Seeds were then plated onto 0.5% agar containing 2.1 g L$^{-1}$ Murashige and Skoog (MS) salts (PhytoTechnology Laboratories) pH 5.7, 1% sucrose and 6.4 g mL$^{-1}$ MS salts vitamin powder (PhytoTechnology Laboratories). Plates were then vernalized at 4°C for at least two days before being moved into the Percival and grown with a 11/13-hour day/night cycle at 22°C. These conditions were also used during seedling infection and PAMP elicitation. The atl6-1 (Salk_083652), atl6-2 (Salk_134489), atl31-1 (GK-746D08), and mkp1 mutants were all in the Columbia (Col-0) background and have been described previously (Bartels et al., 2009; Sato et al., 2009) (Figure 2.2). The atl6-1 mkp1 double mutant was previously generated in the lab by Lingyan Jiang (see thesis for details; Figure 2.13).

Seedling Psuedomonas Growth Measurements

At least four days prior to infection, a glycerol stock stored at -80°C of Psuedomonas syringae pv. tomato DC3000 (Pst DC3000), Pst DC3000 (hrcC), or Pst DC3000ΔavrPto/avrPtoB was streaked onto King’s B (KMB) agar plates containing 30 µg mL$^{-1}$ rifampicin (Pst DC3000 or Pst DC3000 (hrcC)) or 30 µg mL$^{-1}$ rifampicin and 50 µg mL$^{-1}$ kanamycin (Pst DC3000ΔavrPto/avrPtoB) and incubated for two
days at room temperature. A master plate of bacteria was stored at 4°C, and a working plate was streaked two days before infection and grown at room temperature. One day prior to infection, 12-day-old seedlings were aseptically transferred from MS agar plates to 1 mL sterile H₂O in a single well of a 24-well microtiter plate (two seedlings per well). After transferring seedlings, microtiter plates were returned to growth chamber at 21°C overnight for 16-20 hours. For pretreatment experiments, experiments began with 12-day-old seedlings and after initial equilibration overnight, sterile H₂O was replaced with 1 µM flg22 for 8 hours before infection.

Immediately prior to infection, bacterial inoculums were prepared by scraping bacteria off of KMB agar plates and resuspending in sterile H₂O for a final concentration of 2×10⁷ cfu mL⁻¹ (OD₆₀₀=0.02). H₂O or flg22 in microtiter plates was then replaced with the bacterial solution and plates were returned to the growth chamber until sample harvesting. To quantify colony forming units (cfu), serial dilution plating was used three days post-infection. Two seedlings of each genotype were briefly rinsed with sterile H₂O before homogenization in 400 µL of sterile 10 mM MgCl₂. 10-fold dilutions were then prepared with 10 µL being spotted on KMB agar plates containing necessary antibiotics. After two days, colonies were counted, and cfus were calculated.
Adult Plant Pseudomonas Growth Measurements

Pst DC3000 or Pst DC3000ΔavrPto/avrPtoB streaked plates were prepared prior to infection as described above. Bacterial solutions were then prepared by resuspending in sterile H₂O to a final concentration of 1×10⁸ cfu mL⁻¹ (OD₆₀₀=0.1). Silwet was then added for a final concentration of 0.04%. Solution was transferred to a handheld spray bottle and used to infect 4-5-week-old plants. Each plant underwent three rounds of 4 successive sprays from approximately 6-8 inches above the plant. Following inoculation, plants were covered with a transparent dome for the duration of the infection. After three days, colony forming units were quantified by grinding two leaf discs (0.2 cm²) per sample and serial dilution plating as described above.

cAMP Effector Delivery Assay

Two 12-day-old seedlings were transferred from MS agar plates to 1 mL of water in a single well of a 24-well plate and returned to the growth chamber overnight. For pretreatment experiments, sterile water was replaced with 1 mL of 1 µM flg22 for 24 hours before infection. Immediately prior to infection, an inoculum was prepared by scraping DC3000 expressing a fusion of the effector protein AvrPto with adenyllyl cyclase (Pst DC3000 (avrPto-Cya)) (Schechter et al., 2006) from the KMB agar plate and resuspending in sterile H₂O for a final concentration of 2×10⁷ cfu mL⁻¹ (OD₆₀₀=0.02). Infection was carried out by removing H₂O in microtiter plate wells and replacing with 1 mL of bacterial
solution. After twenty-four hours, seedlings were rinsed in sterile H₂O, dried, and flash frozen in liquid nitrogen. Samples were then ground in 0.1 M HCl (100 µL/seedling) and spun at 4°C for 20 minutes at maximum speed. cAMP amount in supernatant was determined by direct cAMP ELISA kit (Enzo Life Sciences). Two to six seedlings were pooled as n=1, and total n=6 for one biological replicate.

**ROS Assays**

Leaf disks (0.2 cm²) were taken from the fully expanded leaves of 5-week-old Arabidopsis plants and cut in half, with each half being an individual sample. Each half disc was floated adaxial side up in 100 µL sterile H₂O in a single well of a white 96-well plate and returned to growth chamber overnight. After 16-20 hours, H₂O was removed and replaced with 100 µL of water containing 200 µM luminol (Sigma, dissolved in 0.2 M KOH), 20 µg mL⁻¹ horseradish peroxidase (Sigma) and elicitors. Luminescence from each well was measured using a Veritas microplate luminometer (Promega).

**Seedling Growth Inhibition by flg22**

6-day-old MS plate-grown seedlings were rinsed and then transferred into 24-well plates (1 seedling per well) containing 1 mL of liquid MS medium (2.1 g L⁻¹ MS salts, pH 5.7, and 1% sucrose) with or without varying concentrations of flg22 (100 nM or 10 µM). After 12 days, seedlings were rinsed in sterile H₂O, patted dry, and weighed individually.
Transcript Accumulation Analysis by qRT-PCR

11-12-day-old seedlings were transferred from MS plates, rinsed in sterile H$_2$O, and placed into 24-well plates containing 1 mL of sterile H$_2$O (two seedlings per well). After equilibration overnight, solution was removed and replaced with 1 µM flg22. At indicated timepoints, plants were removed from wells, briefly rinsed, dried, and flash frozen in liquid N$_2$ (four seedlings per sample). TRIzol reagent (Sigma) and chloroform were used to isolate total RNA which was then precipitated with 100% isopropanol overnight at -20° C. RNA was pelleted and washed with 70% ethanol (made with 200-proof ethanol) before treatment with DNase I (Fermentas) at 37° C to remove DNA contamination. 1 µg of DNase-treated RNA was used to synthesize cDNA in 25 µL reactions containing 5 mM DTT, 0.5 µL RnaseOUT (Invitrogen), 2 µM oligo(dT), 1 mM each of dNTPs and 0.5 µL M-MLV reverse transcriptase (Promega) at 42° C for 1 hour before heat-killing the enzymes at 85° C for five minutes. cDNA samples were then diluted with 25 µL diethylpyrocarbonate (DEPC)-treated H$_2$O for qRT-PCR. 20 µL real-time PCR reactions were performed using 10 µL SYBR Green PCR mix (ABclonal), 2 µL cDNA, and 0.4 µL of each primer in a ABI7500 real-time thermal cycler (Applied Biosystems). Expression levels were calculated using the equation: expression level = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is the difference in Ct values from the gene of interest and the reference gene, and the elicited samples and the unelicited. At2g28390 (SAND family protein) was used as the reference gene for normalization (Czechowski et al., 2005).
Microsomal Isolation and ATL6 Immunoblot Analysis

Microsomal isolation was adapted from Zhang and Peck 2011 (Zhang and Peck, 2011). Six 12-day-old seedlings were flash frozen in liquid N\textsubscript{2} before being ground and homogenized in buffer H (250 mM sucrose, 50 mM HEPES-KOH pH 7.5, 5% glycerol, 50 mM NaPP, 1 mM Na\textsubscript{2}MoO\textsubscript{4}, 25 mM NaF, 10 mM EDTA, 0.5% polyvinyl pyrrolidone). Samples were spun at 4\textdegree C at 21,000 \times g for 10 minutes. Samples were transferred to fresh tubes to remove debris and spun again at 4\textdegree C 21,000 \times g for 20 minutes. Samples were transferred to micro ultracentrifuge tubes and spun at 100,000 \times g for 30 minutes at 4\textdegree C. Supernatant was removed, and pellets were resuspended in 20 µL 4x sample buffer (5% SDS, 10% Glycerol, 0.1 M Tris-HCl, pH 6.8). 15 µL was loaded into each well of a 15% SDS-PAGE gel before transfer to a PVDF membrane for immunoblot analysis. Immunoblotting was performed using αATL6 (1:5000) and αCNX1/2 (1:5000) antibodies diluted in 5% milk in PBST. Chemiluminescence-based detection (Pierce) was performed using horseradish peroxidase-conjugated goat anti-rabbit antibody (Cell Signaling Technologies).

FLS2 Degradation Analysis

2 leaf discs (0.2 cm\textsuperscript{2}) were taken from 4-5-week-old plants and sliced into 5 strips per leaf disc and placed in microcentrifuge tubes with 1 mL of sterile H\textsubscript{2}O overnight. Water was removed and replaced with a 10 µM flg22 solution for one hour. Samples were flash frozen in liquid N\textsubscript{2} before being ground up and
resuspended in 20 µL of 4x sample buffer (as described above). 15 µL of each sample was loaded into each well of a 10% SDS-PAGE gel and transferred to a PVDF membrane. Immunoblot analysis was carried out using αFLS2 and αMPK6 antibodies (1:500 and 1:7500 dilutions in 5% milk in PBST, respectively). Chemiluminescence-based detection was carried out as described above.

**MAPK De-Sensitization Assay**

12-day-old MS-grown seedlings were briefly rinsed and transferred into 1 mL of sterile H₂O in a 24-well plate (2 seedlings per well). After equilibration overnight, water was removed and replaced with 10 µM elf18 or mock solution (sterile H₂O) for one hour. After initial treatment, solutions were removed and replaced with mock solution, 10 µM elf18, or 1 µM pep1 for ten minutes. After second elicitation, samples were flash frozen in liquid N₂. Samples were then ground under liquid N₂ and resuspended in 50 µL 2xSB. 20 µL of each sample was loaded into each well of two 12% SDS-PAGE gels (one for MPK6 levels and the other for MAPK activation) and transferred to PVDF membranes. Immunoblot analysis was carried out using αMPK6 (1:7000) and αpTEpY (1:1000) antibodies diluted in 5% milk in PBST. Chemiluminescence-based detection as carried out as described above.
Table 2.1: Sequences of primers used for PCR genotyping of T-DNA insertion mutants.

| Mutant  | Forward Primer | Reverse Primer | WT | Mutant
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<tr>
<td>ATL6-1 RP</td>
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<tr>
<td>LBb1.3</td>
<td>ATTTTGCAGTTTCCGAAC</td>
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<tr>
<td>WT – ATL6-1 FP and ATL6-1 RP</td>
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<tr>
<td>Mut</td>
<td>ATL6-1 FP and LBb1.3</td>
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<td><strong>atl6-2 (SALK_134489)</strong></td>
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<td>Mut</td>
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**Table 2.2: Sequences of primers used for RT-PCR and qRT-PCR**

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<td>For - ACAAGCTATGGGAAGAAG Rev - TGTCTTTGCACGCAGAC</td>
</tr>
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<td>Reference</td>
<td>AT2G28390</td>
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CHAPTER 3
MKP1 functions as a negative regulator of effector-triggered immunity

This chapter is a draft of a manuscript that will be submitted after a few pathology experiments are completed.

Introduction

When a plant encounters a pathogenic bacterium, it recognizes the presence of a perceived potential threat and initiates downstream responses to stop the infection. The plant uses plasma membrane (PM)-localized pattern recognition receptors (PRRs) to perceive non-self molecules, known as pathogen-associated molecular patterns (PAMPs). After initial perception, the plant initiates a coordinated set of defense responses, known collectively as pattern-triggered immunity (PTI). The goal of one or more of these responses is to limit bacterial growth and avoid development of disease symptoms.

While the plant is utilizing non-self-recognition to initiate defense responses, the bacterium is simultaneously utilizing its own non-self-recognition strategies to detect the potential host to initiate its virulence programs (Yan et al., 2019; O’Malley et al., 2019). Upon recognition, bacteria induce their T3SS and attempt to inject effector proteins into the plant cell. Some of these effectors target
the PTI machinery in the plant to suppress the host’s defense responses and to promote nutrient acquisition (Collmer et al., 2009; Hogenhout et al., 2009).

If the bacteria successfully inject their effector proteins to suppress PTI, another layer of resistance known as effector-triggered immunity (ETI) can be activated to possibly re-establish resistance. Plants have evolved resistance proteins (R genes) to recognize the effectors themselves or their activity, which leads to a more extreme defense response, often resulting in cell death (Cui et al., 2015). Different bacterial effectors target distinct plant components in different parts of the cell. For example, the effector AvrRpm1 leads to phosphorylation of RIN4 at the PM; and this modification of RIN4 is then recognized by the specific R protein, RPM1, leading to an ETI response (Mackey et al., 2002; Boyes et al., 1998). The presence of another effector, AvrRps4, then causes RPS4 nuclear accumulation and initiates an ETI defense response (Wirthmueller et al., 2007). Therefore, while the bacterial injection of T3SS effectors can promote infection in the absence of the cognate R genes, it may result in the induction of a strong resistance in the plant if the host has the correct R genes.

An important aspect of PTI is the restriction of T3SS effector delivery, as this strategy prevents bacterial suppression of resistance or promotion of disease symptoms (Crabill et al., 2010). An understanding of how the plant achieves this restriction came from previous work in the lab studying knockout mutant of MKP1 (mkp1) which is more resistant to pathogen infection (Anderson et al., 2011). The extracellular levels of T3SS-inducing metabolites were lower in mkp1 plants,
resulting in a large decrease in the induction of the T3SS and, therefore, a large decrease in effector delivery into the plant cells (Anderson et al., 2014). Adding back the T3SS metabolites restores the bacteria’s ability to deliver the T3SS effectors and eliminates resistance, demonstrating the extracellular abundance of these chemical signals is necessary and sufficient to explain the resistance in mkp1 (Anderson et al., 2014). Importantly, adding back these metabolites also eliminated the restriction of effector delivery in plants treated with a PAMP to induce PTI (Anderson et al., 2014), indicating that control of the extracellular levels of these chemicals is also the likely explanation for how plants restrict effector delivery during PTI.

Because knocking out MKP1 can result in what seems to mimic a PTI-pre-induced state, it seems likely that there must be a penalty for this apparently advantageous phenotype. Otherwise, it would be difficult to explain why this mutation has not occurred in nature. We hypothesized that a possible explanation is a trade-off with a robust ETI response in the mutant. To initiate an effective ETI response, a plant must recognize the effectors that have been translocated into the plant cell. Because mkp1 plants restrict effector delivery to promote PTI-associated resistance, the decrease in effector delivery may compromise the plant’s ability to mount a robust ETI response. In this work, I used ETI-triggering strains of Pst DC3000 to determine if the absence of MKP1 alters ETI. Surprisingly, MKP1 seems to function as a negative regulator of ETI, with mkp1 plants showing increased resistance to ETI-triggering bacterial strains. Furthermore, this novel phenotype in
*mkp1* is independent of the genetic requirements for the enhanced PTI, indicating that MKP1 is involved in regulation of an independent, yet undefined, pathway during ETI. These results provide one of the few examples for which the function of a protein shared by PTI and ETI can be genetically separated.

**Results**

*mkp1* knock out plants accumulate less PR1 transcript during an ETI-related defense response

Mutant plants lacking MKP1 show increased resistance against the normally virulent bacterial pathogen, *Pst* DC3000, indicating MKP1 is a negative regulator of defense against bacteria (Bartels *et al.*, 2009; Anderson *et al.*, 2011). *mkp1* knock out plants restrict pathogen growth by limiting the translocation of *Pseudomonas syringae* effector proteins into the plant cell through a process that appears to mimic the response of a plant with pre-induced PTI responses (Anderson *et al.*, 2014). As this mode of defense would appear to be advantageous to the plant, a possible reason for why plants have not adopted this strategy more broadly (i.e., why they have not eliminated the *MKP1* gene) is that there is a negative trade-off for the enhanced PTI-like resistance. One possibility is that decreased effector delivery may have negative consequences on another layer of defense, effector-triggered immunity (ETI).

As a first step in testing this possibility, I examined if a molecular response associated with ETI is altered in *mkp1* mutants. ETI-mediated resistance requires
the production of and response to the defense hormone, salicylic acid (SA) (Vlot et al., 2009) The accumulation of transcripts for Pathogenesis-Related Protein 1 (PR1; primers listed in Table 3.1) is often used as a molecular marker for SA-induced responses (Cao et al., 1994; Glazebrook et al., 1996; Uknes et al., 1992). Thus, to examine if SA-induced responses associated with ETI were altered, I surface inoculated 12-day-old Ws and mkp1 (Ws) seedlings with Pst DC3000 expressing the effector protein AvrRpm1 [Pst DC3000 (AvrRpm1)] for 0, 24, and 48 hours before isolating RNA and quantifying PR1 transcripts. As expected, Ws plants accumulated PR1 transcripts at both 24 and 48 hours, increasing with the duration of the infection (Figure 3.1A). mkp1 (Ws) plants also accumulated PR1 transcripts, but to significantly lower levels than observed in wild type plants (Figure 3.1A). These results indicated that mkp1 plants may be compromised in their ability to manifest a robust ETI response due to reduced SA-related signaling.

**mkp1 plants are more resistant to ETI-inducing bacterial strains than wild-type plants**

To determine if mkp1 plants are compromised in their ability to mount an effective ETI response, I surface inoculated Ws (WT) and mkp1 (Ws) knockout plants with Pst DC3000 (AvrRpm1). As expected, when infected with the virulent Pst DC3000, mkp1 (Ws) plants showed reduced bacterial growth compared to WT (Figure 3.1B), demonstrating the increased resistance against virulent pathogens associated with restricted effector delivery. When WT plants were infected with
Pst DC3000 (AvrRpm1), bacterial growth was reduced as compared to infection with Pst DC3000, indicating an ETI-associated defense response (Figure 3.1B). Unexpectedly, when mkp1 (Ws) plants were infected with Pst DC3000 (AvrRpm1), bacterial levels were even further reduced than in WT, indicating a more robust ETI response compared to WT plants (Figure 3.1B).

Different bacterial effectors or their activity are recognized by distinct proteins in the plant cell, often in separate sub-cellular locations. For example, AvrRpm1 leads to the phosphorylation of the Arabidopsis protein RIN4 which is subsequently recognized by the PM-localized RPM1 and leads to an ETI-associated defense response (Mackey et al., 2002; Boyes et al., 1998). In contrast, AvrRps4 targets the NLR pair of RRS1 and RPS4 which results in nuclear accumulation of RPS4 and a subsequent ETI response (Wirthmueller et al., 2007).

To determine if mkp1 plants show enhanced resistance specifically to AvrRpm1 or also to other effectors, I infected WT and mkp1 (Ws) plants with Pst DC3000 expressing the effector AvrRps4 [Pst DC3000 (AvrRps4)]. Fewer bacteria grew on WT plants after infection with Pst DC3000 (AvrRps4) as compared with the virulent strain, indicating an ETI-associated defense response (Figure 3.1C). Consistent with the results from the PM-localized ETI response, fewer bacteria grew on mkp1 (Ws) plants after infection with Pst DC3000 (AvrRps4). These results clearly demonstrate that ETI is not compromised in mkp1 (Ws) mutants even though they accumulate less transcript of the SA-signaling marker PR1. Moreover,
MKP1 is revealed to act as novel, negative regulator of ETI-associated resistance that is initiated in different cellular locations.
Figure 3.1: Despite decreased PR1 levels, mkp1 plants are more resistance to ETI-inducing DC3000 strains

(A) 12-day-old seedling were infected with a 2×10^7 cfu mL^-1 solution (OD_{600} = 0.02) of Pst DC3000 (AvrRpm1) in 24-well plates. At the times indicated, samples were frozen in liquid N\textsubscript{2}. RNA was then isolated, treated with DNase I, and used for cDNA synthesis. qRT-PCR was then performed using primers found in Table 3.1. Values are means ± SEM (n=3), representative of 3 independent experiments. No significant differences were detected within individual time points using a t-test.

(B and C) 12-day-old seedling were immersed in 2×10^7 cfu mL^-1 solution (OD_{600} = 0.02) of Pst DC3000 and Pst DC3000 (AvrRpm1) (B) or Pst DC3000 (AvrRps4) (C) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H\textsubscript{2}O, and ground in 10 mM MgCl\textsubscript{2} before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences detected using a two-way ANOVA test, P<0.0005.
**mkp1 resistance to ETI-triggering bacterial strains is independent of MPK6**

One possible explanation for the decrease in bacterial growth seen in *mkp1* (Ws) in Figure 1 is that the enhanced resistance seen previously in *mkp1* (Ws) mutants is being layered over an ETI response, resulting in an additive decrease. The enhanced resistance against virulent *Pst* DC3000 in *mkp1* (Ws) plants as well as the restriction of effector delivery is dependent upon its direct substrate, MAP Kinase 6 (MPK6) (Anderson *et al.*, 2011; Anderson *et al.*, 2014). Additionally, all PTI-associated molecular phenotypes seen in *mkp1* (Ws) plants are suppressed in *mkp1 mpk6* (Ws) double mutants (Anderson *et al.*, 2011). To determine if MPK6 is similarly required for the enhanced ETI, I infected *mkp1 mpk6* (Ws) double mutants with the ETI-triggering strains of *Pst* DC3000. Consistent with previous results, when infected with *Pst* DC3000, *mkp1* (Ws) plants showed decreased bacterial growth that was lost in the *mkp1 mpk6* (Ws) double mutant (Figure 3.2A). However, when infected with *Pst* DC3000 (AvrRpm1), the enhanced ETI resistance in *mkp1* (Ws) plants was not suppressed in the *mkp1 mpk6* (Ws) double mutant, indicating the resistance is independent of MPK6 (Figure 3.2A). I also infected the double mutant with *Pst* DC3000 (AvrRps4); and both the *mkp1* (Ws) and *mkp1 mpk6* (Ws) plants showed a similar increase in ETI resistance, demonstrating that the enhanced resistance to both ETI-triggering strains of *Pst* DC3000 do not function through MPK6 (Figure 3.2B). Therefore, even in the absence of the MPK6-dependent enhanced PTI-associated resistance against *Pst* D3000, the *mkp1* (Ws) mutants showed the same enhanced ETI resistance. Taken together, these results
indicate that MKP1 functions as a negative regulator of ETI in a previously undescribed pathway that is not related to the restriction of effector delivery.
Figure 3.2: Resistance to ETI-inducing strains of *Pst* DC3000 in *mkp1* plants is independent of MPK6

(A and B) 12-day-old seedling were immersed in 2×10^7 cfu mL\(^{-1}\) solution (OD\(_{600}\) = 0.02) of *Pst* DC3000 and *Pst* DC3000 (AvrRpm1) (A) or *Pst* DC3000 (AvrRps4) (B) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H\(_2\)O, and ground in 10 mM MgCl\(_2\) before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences detected using a two-way ANOVA test, P<0.0005.
**mkp1 plants are resistant to ETI-triggering bacterial strains in Col-0**

To determine if MKP1 plays a role in ETI-associated defense regardless of ecotype, a similar set of pathogen infection assays as described above was carried out in Col-0. First, Col-0, *mkp1* (Col-0), and *mkp1 mpk6* (Col-0) plants were infected with *Pst* DC3000 and *Pst* DC3000 (AvrRpm1). As was observed in the Ws background, while the enhanced *mkp1* (Col-0) resistance to virulent *Pst* DC3000 was suppressed in the *mkp1 mpk6* (Col-0) double mutant, the enhanced resistance to *Pst* DC3000 (AvrRpm1) was not affected by the loss of MPK6, further supporting that MKP1 is functioning in a distinct pathway during an ETI-associated defense response (Figure 3.3A). Similarly, *mkp1* (Col-0) showed lower bacterial growth when infected with *Pst* DC3000 (AvrRps4) as compared to Col-0 plants, demonstrating that the increased ETI also occurs when elicited from different cellular locations in Col-0 (Figure 3.3B). Together, these results show that the novel role for MKP1 in ETI is not unique to the Ws ecotype.
Figure 3.3: Resistance to ETI-inducing strains of *Pst* DC3000 in *mkp1* plants is independent of ecotype

(A and B) 12-day-old seedling were immersed in 2×10⁷ cfu mL⁻¹ solution (OD₆₀₀ = 0.02) of *Pst* DC3000 and *Pst* DC3000 (AvrRpm1) (A) or *Pst* DC3000 (AvrRps4) (B) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H₂O, and ground in 10 mM MgCl₂ before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences detected using a two-way ANOVA test, P<0.0005.
Enhanced ETI resistance in *mkp1* does not require ATL6

The resistance observed in *mkp1* knockout plants to *Pst* DC3000 is known to be dependent on only two proteins so far, its direct substrate, MPK6 (Anderson *et al.*, 2011) and ATL6 (Figure 2.13). Although the resistance to *Pst* DC3000 (AvrRpm1) is independent of MPK6, I wanted to further investigate if other proteins required for the enhanced PTI-resistance in *mkp1* are involved in the ETI-related resistance. Col-0, *mkp1* (Col-0), *atl6-1*, and *mkp1 atl6-1* plants were surface inoculated with *Pst* DC3000 and *Pst* DC3000 (AvrRpm1). As seen in Figure 2.13, when infected with *Pst* DC3000, *mkp1* (Col-0) plants showed increased resistance against the pathogen compared to Col-0; and this resistance was suppressed in the *mkp1 atl6-1* double mutant, confirming that the resistance of *mkp1* (Col-0) plants to *Pst* DC3000 is dependent on ATL6 (Figure 3.4 Left). Examining ETI resistance, *mkp1* (Col-0) plants infected with *Pst* DC3000 (AvrRpm1) showed decreased bacterial levels compared to Col-0, indicating a more robust ETI-associated defense response. However, *mkp1 atl6-1* double mutant plants also showed lower levels of infection, indicating that, in contrast to resistance against *Pst* DC3000, the enhanced ETI resistance seen in *mkp1* (Col-0) plants is independent of ATL6 when infected with the ETI-triggering strain (Figure 3.4 Right). Together with the MPK6 results, these data indicate that MKP1 is functioning in unique pathway from that utilized for the enhanced PTI-associated resistance.
Figure 3.4: MKP1 functions independently of ATL6 during an ETI response.

12-day-old seedling were immersed in a $2 \times 10^7$ cfu mL$^{-1}$ solution (OD$_{600}$ = 0.02) of Pst DC3000 (AvrRpm1) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences detected within individual time points using a two-way ANOVA test, P<0.0001.
**Enhanced ETI resistance in *mkp1* does not require MPK3**

Because the resistance in *mkp1* mutants is independent of the previously described genetic pathways, I decided to try to identify other potential proteins acting in the MKP1-dependent ETI pathway. Previous work has shown that while MPK3 interacts with MKP1 in a directed yeast-two hybrid, it is not required for *mkp1*-associated resistance to *Pst* DC3000 (Ulm *et al.*, 2002). To determine if MPK3 plays a role in the increased resistance of *mkp1* (Col-0) plants to ETI-triggering *Pst* DC3000 strains, *mkp1* mpk3 double knockouts were infected with *Pst* DC3000 and *Pst* DC3000 (AvrRpm1). As previously seen (Anderson *et al.*, 2011), when infected with *Pst* DC3000, *mkp1* (Col-0) plants showed increased resistance that was not suppressed in the *mkp1* mpk3 double mutants, confirming that the enhanced resistance to *Pst* DC3000 in *mkp1* does not require MPK3 (Figure 3.5 Left).

Similarly, when infected with *Pst* DC3000 (AvrRpm1), fewer bacteria grew on *mkp1* (Col-0) plants when compared to Col-0. Because the enhanced resistance was still observed in *mkp1* mpk3 plants, the enhanced resistance to *Pst* DC3000 (AvrRpm1) in *mkp1* (Col-0) is also independent of MPK3 (Figure 3.5 Right).
Figure 3.5: MKP1 functions independently of MPK3 during an ETI response.

12-day-old seedling were immersed in a $2 \times 10^7$ cfu mL$^{-1}$ solution ($\text{OD}_{600} = 0.02$) of Pst DC3000 (AvrRpm1) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences detected within individual time points using a two-way ANOVA test, P<0.0001.
**Enhanced ETI resistance in mkp1 does not require CDPKs**

After determining that MKP1 is functioning in a previously undescribed pathway during an ETI response, and is not functioning with MPK3, I checked to see if MKP1 functions with the calcium-dependent protein kinases (CDPKs) in an ETI-defense response. MKP1 contains two calmodulin-binding domains (CaMBD) and has been shown to interact with and be regulated by calmodulins (Lee *et al.*, 2008; Yamakawa *et al.*, 2004). CDPK5/6/11 play a role in regulating PTI responses in *Arabidopsis* (Boudsocq *et al.*, 2010). Additionally, previous work in the lab has shown that some defense-related transcripts that hyperaccumulate in *mkp1* (Col-0) plants are dependent on the CDPKs (Ying Wan thesis). To investigate whether the CDPKs are required for *mkp1*-associated resistance to ETI-triggering strains of *Pst* DC3000, I used a *mkp1 cdpk5/6/11* quadruple knockout mutant previously generated in the lab (Ying Wan thesis). When infected with *Pst* DC3000, *mkp1* (Col-0) plants show enhanced resistance which is not suppressed in the *mkp1 cdpk5/6/11* quadruple mutant, confirming that *mkp1*-associated resistance to *Pst* DC3000 is independent of the CDPKs (Figure 3.6 Left). Similarly, when infected with *Pst* DC3000 (AvrRpm1), the decreased bacterial levels in *mkp1* (Col-0) plants were also reduced in *mkp1 cdpk5/6/11* plants, indicating that *mkp1* resistance to *Pst* DC3000 (AvrRpm1) is also independent of the CDPKs (Figure 3.6 Right).
Figure 3.6: MKPI functions independently of the CDPKs during an ETI response.

12-day-old seedling were immersed in a $2 \times 10^7$ cfu mL$^{-1}$ solution (OD$_{600} = 0.02$) of Pst DC3000 (AvrRpm1) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences detected within individual time points using a two-way ANOVA test, P<0.05.
Enhanced ETI resistance in \textit{mkp1} is dependent on SID2

Because of its importance to ETI, I decided to investigate the role of SA in the enhanced resistance in \textit{mkp1}. Because \textit{mkp1} plants are more resistant to ETI-triggering \textit{Pst} DC3000 strains despite accumulating less \textit{PRI} transcripts, I hypothesized that \textit{mkp1} resistance would be independent of SA-associated proteins. SA-Induction Deficient 2 (\textit{sid2}) knockout mutants do not produce isochorismate synthase, an enzyme necessary for the biosynthesis of SA, resulting in increased susceptibility to \textit{Pst} DC3000 (Nawrath and Méraux, 1999; Wildermuth \textit{et al.}, 2002; Chen \textit{et al.}, 2009). To determine if SID2 is involved in \textit{mkp1}-associated ETI resistance, \textit{mkp1 sid2} double mutants were infected with \textit{Pst} DC3000 (AvrRpm1). The resistance observed in \textit{mkp1} (Col-0) is suppressed in the \textit{mkp1 sid2} mutant, indicating that SID2 is required for the resistance to \textit{Pst} DC3000 (AvrRpm1) in \textit{mkp1} plants (Figure 3.7). This indicates that SA is required for \textit{mkp1}-associated resistance to ETI-triggering DC3000 strains.
Figure 3.7: MKP1 resistance during an ETI response is dependent upon SID2.

12-day-old seedling were immersed in a $2 \times 10^7$ cfu mL$^{-1}$ solution (OD$_{600}$ = 0.02) of Pst DC3000 (AvrRpm1) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences detected within individual time points using a one-way ANOVA test, P<0.001.
MKP1 is a negative regulator of ETI in soil-grown and adult plants

All previous experiments were carried out as passive infections of young seedlings grown on agar plates before transferring to water in 24-well plates for infections. To determine if the enhanced ETI resistance in *mkp1* can also be observed in soil grown plants, 3-week-old, soil-sown Col-0 and *mkp1* (Col-0) plants were spray inoculated with *Pst* DC3000 and *Pst* DC3000 (AvrRpm1). After three days of infection with *Pst* DC3000, *mkp1* (Col-0) plants showed significantly decreased bacterial levels compared to WT, indicating the same enhanced PTI resistance is observed in soil grown seedlings (Figure 2.8A). Similarly, when infected with *Pst* DC3000 (AvrRpm1), Col-0 plants showed significant ETI-induced resistance compared to infection with *Pst* DC3000, and *mkp1* (Col-0) plants showed even more resistance. These experiments show that the enhanced ETI resistance in *mkp1* is not restricted to seedlings infected in the microtiter plate system.

I also wanted to determine if MKP1 acts as a negative regulator of ETI in adult soil grown plants. Five-week-old Ws and *mkp1* (Ws) plants were syringe infiltrated (Figure 3.9A) with *Pst* DC3000 or *Pst* DC3000 (AvrRpm1); and bacterial growth was measured three days post-infection. When infected with *Pst* DC3000, *mkp1* (Ws) plants showed slightly decreased, although not statistically significant, bacterial levels compared to WT, indicating the enhanced PTI resistance is also observed in adult leaves using syringe infiltration. More replicates will be needed to establish statistical significance. When infected with *Pst* DC3000 (AvrRpm1),
Ws plants showed decreased bacterial levels compared to the fully virulent pathogen, indicating a successful ETI defense response. Similar to what was observed in seedlings, \textit{mkp1} (Ws) plants showed a further decrease in bacterial levels compared to WT plants, suggesting enhanced resistance to ETI triggering strains of \textit{Pst} DC3000 (Figure 3.8B). These result shows that the enhanced ETI resistance in \textit{mkp1} also occurs in adult plants using syringe infiltration.

To determine if the enhanced resistance is also independent of MPK6 in adult plants, Col-0, \textit{mkp1} (Col-0), and \textit{mkp1 mpk6} (Col-0) plants were infiltrated with \textit{Pst} DC3000 and \textit{Pst} DC3000 (AvrRpm1). As expected, when infected with \textit{Pst} DC3000, the resistance seen in \textit{mkp1} (Col-0) plants was suppressed in the \textit{mkp1 mpk6} (Col-0) double mutants, indicating MPK6 is required for \textit{mkp1} resistance. In contrast, the resistance to \textit{Pst} DC3000 (AvrRpm1) seen in \textit{mkp1} (Col-0) plants was not suppressed in the double mutants, indicating that, as in seedlings, \textit{mkp1} resistance to ETI-triggering strains of \textit{Pst} DC3000 is independent of MPK6, functioning in an undescribed pathway (Figure 3.9C). Together, these data reinforce the importance of MKP1 as a novel negative regulator of ETI in multiple stages of plant development. Moreover, they demonstrate that results from the agar-grown seedling assays are predictive of resistance observed in adult plants.
Figure 3.8: MKP1 is a negative regulator of ETI in soil-grown plants.

3-week-old soil sown seedlings were spray inoculated with a $2 \times 10^8$ cfu mL$^{-1}$ solution ($\text{OD}_{600} = 0.2$) of *Pst* DC3000 (AvrRpm1). 3 dpi, seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ before serial dilution plating with 8 samples per experiments. Values are means ± SEM (n=24) pooled from 3 independent experiments. Letter groups indicate significant differences determined using a two-way ANOVA test, $P<0.001$. 
Figure 3.9: MKP1 is a negative regulator of ETI-associated resistance in adult plants.

(A) Picture showing infiltrated leaf, showing two holes each at the base and tip of the leaf on either side of the midvein. Leaves were infiltrated from the adaxial surface. (B and C) 4-5-week-old plants were infiltrated as described above with a $5 \times 10^5$ cfu mL$^{-1}$ solution ($OD_{600} = 0.0005$) of Pst DC3000 (AvrRpm1). 3 dpi, leaf punches (0.2 cm$^2$) were taken from infected tissue (two per leaf) and ground in 10 mM MgCl$_2$ before serial dilution plating with 4-8 samples per experiment. Values are means ± SEM (B: n=12; C: n=6) with Letter groups indicate statistical significance using a two-way ANOVA. $P<0.001$ (B), $P<0.01$ (C). Experiment was performed twice (B) or once (C).
Discussion

**MKP1 is a novel regulator of ETI**

Plants lacking MKP1 have previously been shown to be more resistant to infection with virulent *Pst* DC3000 (Anderson et al., 2011). This resistance is due to the plant’s ability to decrease the levels of extracellular metabolites required by the bacteria to induce its type III secretion system, resulting in a large decrease in the amount of effector proteins injected into the plant cell (Anderson et al., 2014). Due to the decrease in effector delivery, I hypothesized that perhaps a tradeoff for this form of enhanced resistance would be a decrease in the plant’s ability to mount an effective ETI response because of the lower concentration of effectors in the plant cell. Surprisingly, after infection with ETI-triggering strains of *Pst* DC3000, *mkp1* plants show enhanced resistance, indicating that *mkp1* plants are not impaired in their ability to trigger ETI, but they are more effective. These results indicate that MKP1 is negative regulator of ETI.

MKP1 dephosphorylates MPK6 to negatively regulate the activity of the MAPK; and MPK6 is required for the restriction of pathogen growth and effector delivery in *mkp1* plants (Anderson et al., 2011; Anderson et al., 2014). Interestingly, when infected with ETI-triggering *Pst* strains, *mkp1* resistance is independent of MPK6, meaning that the enhanced ETI is not simply the resistance against *Pst* DC3000 being layered over a general ETI response. Moreover, these results indicate that MKP1 is functioning in a distinct signaling pathway from how it functions during a *Pst* DC3000 infection. This point is further supported by the fact
that the \textit{mkp1}-associated resistance is independent of ATL6 during an ETI response but dependent on ATL6 for the enhanced resistance against \textit{Pst DC3000}, demonstrating that the two mechanisms are genetically distinct.

An interesting additional consideration is that the same level of enhanced ETI resistance occurs even with the very large differences in effector delivery between \textit{mkp1} and \textit{mkp1 mpk6} plants. Because previous experiments indicate that bacteria have > 70\% reduction in effector delivery in \textit{mkp1} plants (Anderson et al., 2014), these results indicate that under normal infection conditions, the pathogen must be delivering amounts of effector proteins far in excess of what the plant requires to trigger an effective ETI response.

**Enhanced ETI resistance in \textit{mkp1} does not require other candidate kinases implicated in both PTI and ETI resistance**

Because the known genetic suppressors of the enhanced PTI resistance in \textit{mkp1} do not suppress this mutant’s enhanced ETI, I investigated the requirement of other potential candidates. MKP1 has been shown to interact with MPK3 in a Y2H (Ulm \textit{et al.}, 2002), and MPK3 is activated in an ETI response (Tsuda \textit{et al.}, 2013). Therefore, it was possible that this kinase plays a more important role in the enhanced ETI in \textit{mkp1} plants. However, the resistance in \textit{mkp1} plants to ETI-triggering strains is not altered in the absence of MPK3. Other candidate kinases came from consideration of MKPI’s domain structure. MKP1 contains two calmodulin binding domains, suggesting that it may play a role in calcium-related
responses. Previous work in the lab showed that some increased accumulation of defense-related transcripts in mkp1 plants was dependent on a set of CDPKs (CDPK 5/6/11) (Ying Wan’s thesis). Furthermore, knocking out these CDPKs has been reported to partially compromise ETI (Gao et al., 2014). Therefore, I investigated if these partially redundant kinases are required for the enhanced ETI resistance in mkp1 cpk5 cpk6 cpk11 using the quadruple mutant previously produced in our lab. For either wild-type Pst DC3000 or the ETI-triggering strains, the absence of the CDPKs did not affect the enhanced resistance in mkp1. Therefore, an important question moving forward is what signaling pathway(s) is function in the pathway that leads to mkp1 resistance during ETI.

**SA is required for the enhanced resistance in mkp1 during ETI**

Salicylic acid (SA) plays an important role in ETI, and mutants lacking key signaling or biosynthetic components are often more susceptible to infection compared to wild type (Vlot et al., 2009; Dempsey et al., 1999). Therefore, it was important to determine if SA is required for the enhanced ETI-associated resistance in mkp1 plants. Previously in the lab, double mutants were generated between mkp1 and sid2, which is required for SA biosynthesis. When infected with wild-type Pst DC3000, mkp1 resistance is independent of SID2, indicating that this mode of resistance does not require SA (Ying Wan thesis). By contrast, enhanced ETI resistance against Pst DC3000 (AvrRpm1) in mkp1 requires SID2. Therefore, even though PR1 transcript accumulation, a molecular marker of SA responses, is
reduced in *mkp1*, the mutants still require SA. These results indicate a potentially complex relationship between the enhanced ETI resistance in *mkp1* and SA. The *mkp1* mutation does not appear to increase SA sensitivity or responsiveness as (a) *PR1* transcript levels are reduced during ETI and (b) *mkp1* plants are actually less responsive to direct SA application (Ying Wan’s thesis). Therefore, it would appear that whatever occurs in the *mkp1* mutant may not act directly through SA but still requires at least some aspect of the defense hormone. If true, MKP1 may play a role in an as-yet undefined synergistic response that may augment ETI as long as SA is produced.

**MKP1 regulates ETI at multiple locations in the cell**

When *Pst* DC3000 injects effector proteins, they have specific host targets in multiple locations within the plant cell, meaning that the host R genes initiate ETI from different cellular locations as well. For example, AvrRpm1 leads to phosphorylation of RIN4 at the plasma membrane (PM) which is recognized by RPM1 at the PM, whereas AvrRps4 causes RPS4 accumulation in the nucleus (Mackey *et al.*, 2002; Boyes *et al.*, 1998; Wirthmueller *et al.*, 2007). Interestingly, MKP1 negatively regulates ETI-associated resistance initiated when *Pst* DC3000 expresses either AvrRpm1 or AvrRps4, indicating that the absence of MKP1 results in enhanced ETI when signaling is initiated from unrelated cellular compartments.

Overall, the results of this chapter report the identification of MKP1 as a novel negative regulator of ETI-associated resistance. Intriguingly, the mechanism
must be distinct from MKPI’s role in PTI as there are very different genetic requirements for the resistance in \textit{mkp1} during PTI vs ETI. This observation is among the first examples of a common component shared by these two types of immunity being genetically separable. Although the mechanism and pathway of action remain unclear for what is being altered in \textit{mkp1}, the future elucidation of the mechanism(s) could add to potential new strategies for enhancing resistance in field crops to promote improved food production.

\textbf{Materials and Methods}

\textbf{Plant Material and Growth Conditions}

\textit{Arabidopsis} seeds were sterilized using a 10\% bleach and 0.05\% Tween20 solution for 15 minutes while spinning before being thoroughly washed with sterile \textit{H\textsubscript{2}O}. Seeds were then plated onto 0.5\% agar containing 2.1 g L\textsuperscript{-1} Murashige and Skoog (MS) salts (PhytoTechnology Laboratories) pH 5.7, 1\% sucrose and 6.4 g mL\textsuperscript{-1} MS salts vitamin powder (PhytoTechnology Laboratories). Plates were then vernalized at 4\textdegree C for at least two days before being moved into the Percival and grown with a 11/13-hour day/night cycle at 22\textdegree C. These conditions were also used during seedling infection. \textit{mkp1} single and \textit{mkp1 mpk6} double mutants were in both the Wassilewskija (Ws) and Columbia (Col-0) backgrounds and have been described previously (Bartels \textit{et al.}, 2009). The \textit{atl6-1 mkp1} double mutant was previously generated in the Col-0 background in the lab by Lingyan Jiang and is described in Chapter 2 (Figure 2.13). The \textit{cdpk5/6/11} triple and the \textit{cdpk5/6/11 mkp1}
quadruple mutants were both previously generated in the Col-0 background by Ying Wan (see thesis for details). *mpk3* and *m kp1 mpk3* mutants were in the Col-0 background and have been described previously (Bartels et al., 2009). *pad4* and *sid2* single mutants as well as the *m kp1 pad4* and *m kp1 sid2* double mutants were generated by Roman Ulm’s lab and were briefly described in Ying Wan’s thesis.

**Passive Infection Seedling **Pseudomonas** Growth Measurements

At least four days prior to infection, a glycerol stock stored at -80°C of *Psuedomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), *Pst* DC3000 carrying ArRpm1 (*Pst* DC3000 (AvrRpm1)), and/or *Pst* DC3000 carrying AvrRps4 (Pst DC3000 (AvrRps4)) was streaked onto King’s B (KMB) agar plates containing 30 µg mL⁻¹ rifampicin (*Pst* DC3000) or 30 µg mL⁻¹ rifampicin and 50 µg mL⁻¹ kanamycin (*Pst* DC3000 (AvrRpm1) and *Pst* DC3000 (AvrRps4)) and incubated for two days at room temperature. A master plate of bacteria was stored at 4°C and a working plate was streaked two days before infection and grown at room temperature. One day prior to infection, 12-day-old seedlings were aseptically transferred from MS agar plates to 1 mL sterile H₂O in a single well of a 24-well microtiter plate (two seedlings per well). After transferring seedlings, microtiter plates were returned to growth chamber at 21°C overnight for 16-20 hours.

Immediately prior to infection, bacterial inocula were prepared by scraping bacteria off KMB agar plates and resuspending in sterile H₂O for a final concentration of 2×10⁷ cfu mL⁻¹ (OD₆₀₀=0.02). H₂O in microtiter plates was then
replaced with the bacterial solution and plates were returned to the growth chamber until sample harvesting. To quantify colony forming units (cfu), serial dilution plating was used three days post-infection. Two seedlings of each genotype were briefly rinsed with sterile H₂O before homogenization in 400 µL of sterile 10 mM MgCl₂. 10-fold dilutions were then prepared with 10 µL being spotted on KMB agar plates containing necessary antibiotics. After two days, colonies were counted and cfus were calculated.

**Spray Infection Pseudomonas Growth Measurements**

After sterilization, *Arabidopsis* seeds were sown directly onto soil, vernalized at 4°C for two days, and grown in a 10/14-day/night cycle at 21°C for 2-3 weeks. Immediately prior to infection, bacterial inocula of *Pst* DC3000 and *Pst* DC3000 (AvrRpm1) were prepared as described above to a final concentration of 2×10⁸ (OD₆₀₀ = 0.2). Three dpi, seedlings were removed from soil, briefly rinsed in sterile H₂O, followed by cfu calculation as described above.

**Infiltration Pseudomonas Growth Measurements**

Immediately prior to infection, bacterial inocula of *Pst* DC3000 and/or *Pst* DC3000 (AvrRpm1) were prepared as described above to a final concentration of 5×10⁵ (OD₆₀₀ = 0.0005). Two small holes were made with sterile gel-loading pipette tips at both the tip and base of 4-5-week-old plant leaves, one on each side of the midvein (Figure 3.3a). Inocula were transferred into a needleless syringe and
infiltrated into the plant leaf’s adaxial side using a gloved finger to create a seal from the abaxial surface at each of the four holes. Three dpi, leaf punches (0.2 cm²) were taken from infected leaves and cfus were calculated (two discs per sample) as described above.

**Transcript Accumulation Analysis by qRT-PCR**

11-12-day-old seedlings were transferred from MS plates, rinsed in sterile H₂O, and placed into 24-well plates containing 1 mL of sterile H₂O (two seedlings per well). After equilibration overnight, solution was removed and replaced with a 2×10⁷ cfu mL⁻¹ (OD₆₀₀=0.02) solution of *Pst* DC3000 (AvrRpm1). At indicated timepoints, plants were removed from wells, briefly rinsed, dried, and flash frozen in liquid N₂ (four seedlings per sample). TRIzol reagent (Sigma) and chloroform were used to isolate total RNA which was then precipitated with 100% isopropanol overnight at -20°C. RNA was pelleted and washed with 70% ethanol (made with 200-proof ethanol) before treatment with DNase I (Fermentas) at 37°C to remove DNA contamination. 1 µg of DNase-treated RNA was used to synthesize cDNA in 25 µL reactions containing 5 mM DTT, 0.5 µL RnaseOUT (Invitrogen), 2 µM oligo(dT), 1 mM each of dNTPs and 0.5 µL M-MLV reverse transcriptase (Promega) at 42°C for 1 hour before heat-killing the enzymes at 85°C for five minutes. cDNA samples were then diluted with 25 µL diethylpyrocarbonate (DEPC)-treated H₂O for qRT-PCR. 20 µL real-time PCR reactions were performed using 10 µL SYBR Green PCR mix (ABclonal), 2 µL
cDNA, and 0.4 µL of each primer in a ABI7500 real-time thermal cycler (Applied Biosystems). Expression levels were calculated using the equation: expression level = $2^{-\Delta \Delta C_t}$, where $\Delta \Delta C_t$ is the difference in $C_t$ values from the gene of interest and the reference gene, and the elicited samples and the unelicited. At2g28390 (SAND family protein) was used as the reference gene for normalization (Czechowski et al., 2005).
Table 3.1: Sequences of primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession Number</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRI</td>
<td>AT2G14610</td>
<td>For – GGAGCTACGCAGAACAACTAAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev - CCCACGAGGATCATAGTTGCAA</td>
</tr>
<tr>
<td>Reference</td>
<td>AT2G28390</td>
<td>For – AACTCTATGCAGCATTTGATCCACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev - TTCAGTTCCTCCCGGTCA</td>
</tr>
</tbody>
</table>
CHAPTER 4

Elucidating other members of the MKP1-dependent PTI pathway

This chapter contains preliminary work that will be continued in the lab by another graduate student as part of their dissertation.

Introduction

After demonstrating that ATL6 plays a novel role in plant defense, I began work on two related topics that, although not yet complete, provide promising evidence that these topics should be investigated further. The first portion is on ATL31, a protein with the highest level of identity and similarity to ATL6 in this gene family. The second short section was the initial work to identify the intervening kinase between MPK6 and ATL6. These experiments will be summarized in this chapter.

I first wanted to determine if ATL31, the gene family member most highly related to ATL6, may have defense-related phenotypes similar to ATL6. Previous work has shown that ATL6 and ATL31 play similar roles in positively regulating the C/N ratio response, suggesting that they may both function in the defense response as well (Sato et al., 2009). Although neither single mutant has previously been found to have a defense-related phenotype, a double knockout mutant (atl6 atl31), displays a slight susceptibility to infection with Pst DC3000, indicating that both may play a related role (Maekawa et al., 2012). In a C/N response, the ATL31-
dependent pathway has been partially elucidated, with some upstream components and substrates identified (Maekawa et al., 2014; Yasuda et al., 2014; Sato et al., 2011; Yasuda et al., 2017). This information provides some valuable insight of candidates to probe for roles in this novel PTI-associated defense pathway.

In this chapter, I describe results that demonstrate that atl31 mutants have similar defense phenotypes as described for atl6. However, at least using our assay conditions, we do not see additive effects in the double mutant, nor do we see enhanced susceptibility to Pst DC3000. Therefore, additional work remains to determine why both single mutants seem to phenocopy each other even though the double mutant does not currently display any enhanced susceptibility. This additional work is in progress as part of the thesis of another graduate student in the lab. In addition, I have found that knocking out the activity of a set of PM CBL-CIPKs phenocopies the atl6 mutant as well, indicating that this kinase family may be the missing component of the signaling pathway connecting MPK6 to ATL6. This work is also being continued by the same graduate student.

Results and Discussion

ATL31 is highly related to ATL6

ATL6 is a positive regulator of PTI (see previous chapter). Although there are over 90 genes in the ATL family in Arabidopsis, ATL31 is the most highly related to ATL6, sharing the same domain structure as well as 65% amino acid identity
(Guzmán, 2012). In addition, ATL31 shares the two flg22-induced phosphorylation sites observed in ATL6 (Figure 4.1A). Interestingly, other ATLs implicated in defense (ATL2, ATL9) based on transcript accumulation after PAMP elicitation (Salinas-Mondragón et al., 1999; Serrano and Guzmán, 2004; Berrocal-Lobo et al., 2010), do not share these phosphorylation sites. Because of the potential conservation of PAMP-induced post-translational regulation, I wanted to determine if ATL31 may also play a role in PTI.
Figure 4.1: ATL6 and ATL31 are highly related proteins and share two phosphorylation sites.

(A) Sequence alignment of ATL6 and ATL31 showing conserved phosphorylation sites. R-X-X-S motif marked denoted with bold and phosphorylation site also underlined. Italicized sequences in ATL2/ATL9 indicate identical/similar amino acids. (B) Sequence alignment of ATL6 and ATL31 with ATL6 antibody sequence in bold.

|  | ATL6  | 268 | MKDWKLNRTNSLVLPRGGSSRGKPIDRSDARSDDRMLFRKTPSLWR |
|  | M     | +WKLNRS+VLPFGSSRGK+DSRARSDDRMLFRKTPSLWR |
|  | ATL31 | 237 | MANWKLNRSNSVVFVLPFGSSRGKQVDRSRAKSDRMLFRKTPSLWR |
|  | ATL2  | 233 | FSEFEDELT----------RRDSPASQS |
|  | ATL9  | 286 | MKT----SRTMGHVALLPQARSSRGY |

|  | ATL6  | 348 | DRWAFLRNASFLWRNSS-VHVPQGGV-NKDGEGT-SV |
|  |      |     | DRWAFLRN SFLWRNS++VPR VNKDGEGT SV |
|  | ATL31 | 315 | DRWAFLRNPSFLWRNTTPVPSPRVEVNNKDGEGTSSV |
|  | ATL2  | 300 | EESR---------- |
|  | ATL9  | 365 | ER--------SFERLPDRV |

|  | ATL6  | 200 | PEPVVTVAPVPEQHLTSEVDSRRLPVPEDVLRKVF |
|  |      |     | PVPV------------------------VELPRVVF |

|  | ATL31 | 192 | PVPV------------------------VELPRVVF |
ATL31 is required for a full PTI response

To examine a potential role for ATL31, a T-DNA insertion atl31 knockout mutant (GK-746D08) was obtained. In addition, to address potential functional redundancy, an atl6-1 atl31 double mutant was generated (Figure 4.2). Col-0, atl6-1, atl31, and atl6-1 atl31 seedlings were passively infected with the virulent pathogen, Pst DC3000; and all genotypes showed similar levels of bacterial growth at 3 dpi (Figure 4.3A left). As Pst DC3000 can strongly suppress PTI, it can often overwhelm the plant’s initial defenses, masking alterations to PTI-associated resistance. To better observe potential changes in PTI in the mutants, the compromised Pst DC3000ΔavrPto/avrPtoB mutant was used. As expected, the compromised bacteria did not grow as well in Col-0 plants as compared to Pst DC3000, indicating a more effective PTI response. In contrast, atl6-1, atl31, and atl6-1 atl31 plants showed infection levels comparable to those seen with Pst DC3000, showing they are impaired in their ability to mount a full PTI response (Figure 4.3A right). Similar results were obtained using spray inoculation of soil grown plants (Figure 4.3B), demonstrating that the observed resistance phenotypes occur using different plant growth conditions. The fact that atl6-1 and atl31 single mutants showed similar susceptibility phenotypes indicates that ATL6 and ATL31 do not function redundantly in PTI. However, because atl6-1 atl31 double knockout plants show the same level of infection as seen in either single mutant, ATL6 and ATL31 may both be required during PTI, perhaps with the two proteins functioning as a dimer. Alternatively, the passive infection method in microtiter
plates and spray inoculation in seedlings may not allow enough bacterial growth
to detect enhanced susceptibility beyond what is observed with *Pst* DC3000. Indeed, although no defense-related phenotype has been reported for either of the single mutants, infiltration of adult *atl6-1 atl31* double mutant plants displayed increased susceptibility to *Pst* DC3000, indicating perhaps ATL6 and ATL31 function redundantly (Maekawa *et al.*, 2012).
Figure 4.2: Generation of the *atl6-1 atl31* double mutant.

The *atl6 atl31* double mutant was confirmed by genotyping plant leaf tissue using primers listed in Table 4.1. *wt*, represents the wild type genes; *mu*, represents the mutated genes with T-DNA insertion; *actin* was used for internal reference. Mutant was generated by Lingyan Jiang.
Figure 4.3: *atl31* and *atl6-1 atl31* mutants are not able to mount an effective PTI-associated defense response.

(A) 12-day-old seedlings were immersed in $2 \times 10^7$ cfu mL$^{-1}$ solution of *Pst* DC3000 and *Pst* DC3000Δ*AvrPto/AvrPtoB* in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ (2 seedlings per sample) before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences using an ANOVA $P<0.0001$. (B) 3-week-old soil sown seedlings were spray inoculated with a $2 \times 10^8$ cfu mL$^{-1}$ solution (OD$_{600} = 0.2$) of *Pst* DC3000 and *Pst* DC3000Δ*AvrPto/AvrPtoB*. 3 dpi, seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ before serial dilution plating with 8 samples per experiments. Values are means ± SEM (n=24) pooled from 3 independent experiments. Letter groups indicate significant differences determined using a two-way ANOVA test, $P<0.05$. 
Loss of ATL31 does not alter PTI-associated growth inhibition

Even though all other PTI-associated responses tested were not altered, atl6 plants are unable to mount an effective PTI response. To begin to examine if atl31 plants behave similarly, I performed a flg22-induced growth inhibition experiment. Seedlings were transferred from plates to liquid MS media with increasing concentrations of flg22 and allowed to grow for 12 days. As expected, all plants showed flg22-induced growth inhibition, with greater inhibition occurring at higher concentrations. atl mutants responded very similarly to Col-0 plants, with only a small but reproducible increase in fresh weight per seedling at 100 nM flg22 in the atl6-1 atl31 double mutants (Figure 4.4). These results indicate that growth inhibition is slightly suppressed compared to Col-0 or the single atl6-1 and atl31 mutants. Therefore, ATL6 and ATL31 appear to function redundantly in a fairly minor contribution to flg22-induced growth inhibition.
Figure 4.4: flg22-induced growth inhibition is unaltered in *atl31* and *atl6-1* *atl31* mutants.

6-day-old plants were placed in 0.5x liquid MS media in 24-well plates (one seedling per well) along with varying concentrations of flg22. After 12 days of elicitation, fresh weight was measured for each individual seedling. Values are means ± SEM (n=54) pooled from 3 independent experiments. Letter groups indicate significant differences calculated from a two-way ANOVA, P<0.05.
**ATL31 and ATL6 do not play a role in ETI**

Both ATL6 and ATL31 are required for a full PTI-associated defense response. To investigate if these proteins are also required for ETI, plants were passively infected with *Pst* DC3000 expressing the effector protein AvrRpm1 [*Pst* DC3000 (AvrRpm1)]. Col-0 plants showed a significant decrease in bacterial levels, indicating an effective ETI response that restricts pathogen growth (Figure 4.5). Similarly, bacteria grew to equivalent levels as in WT in all *atl* knockout mutants. Therefore, ATL6 and ATL31 do not appear to function in ETI-mediated resistance.
Figure 4.5: *atl31* and *atl6-1 atl31* mutants are unaltered in their ability to mount an ETI-associated defense response.

12-day-old seedlings were immersed in $2 \times 10^7$ cfu mL$^{-1}$ solution of *Pst* DC3000 and *Pst* DC3000 (AvrRpm1) in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ (2 seedlings per sample) before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences using an ANOVA $P<0.0001$. 
ATL31 functions downstream of MKP1 in PTI

As the loss of ATL6 or ATL31 have similar effects on PTI-associated resistance, it was plausible that ATL31 may also function downstream of MKP1 as was found for ATL6. To test this possibility, an atlas1 mkp1 double mutant was generated and confirmed through PCR genotyping (Figure 4.6). Col-0, atlas1, mkp1, and atlas1 mkp1 seedlings were passively infected with Pst DC3000. Consistent with the results in Figure 4.3, the virulent pathogen grew to similar levels in both atlas1 and Col-0. In mkp1 plants, however, bacterial growth was significantly reduced, indicating enhanced resistance against the pathogen in this mutant. Interestingly, this enhanced resistance was suppressed in atlas1 mkp1 plants, as the bacterial growth was restored to levels of infection seen in wild-type plants (Figure 4.7). This result indicates that the enhanced resistance seen in mkp1 plants is dependent on ATL31, linking them genetically. Moreover, ATL6 and ATL31 share another similarity in PTI, as they both act downstream of the MKP1-regulated pathway.
Figure 4.6: Generation of the *atl31 mkp1* double mutant.

The *atl31 mkp1* double mutant was confirmed by genotyping plant leaf tissue using primers listed in Table 4.1. *wt*, represents the wild type genes; *mu*, represents the mutated genes with T-DNA insertion.
Figure 4.7: Resistance to *Pst* DC3000 seen in *mkp1* plants is dependent upon ATL31.

12-day-old seedlings were immersed in $2 \times 10^7$ cfu mL$^{-1}$ solution of *Pst* DC3000 and in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ (2 seedlings per sample) before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences using an ANOVA P<0.0001.
Resistance in *mkp1* plants is dependent on ATL6 and/or ATL31

Because ATL6 and ATL31 appear to each be capable of suppressing the enhanced resistance in *mkp1* plants, I wanted to test if there might be an additive effect if both proteins were removed. Making use of an *atl6-1 atl31 mkp1* triple mutant that was previously generated in the lab (Figure 4.8), Col-0, *atl6-1 atl31*, *mkp1*, and *atl6-1 atl31 mkp1* plants were passively infected with *Pst* DC3000. As described above, *atl6-1 atl31* double mutants showed approximately wild-type levels of infection, while *mkp1* plants showed increased resistance against the pathogen. In the *atl6-1 atl31 mkp1* triple mutants, however, wild-type levels of infection were observed, showing a similar level of suppression of the enhanced resistance in *mkp1* plants as seen in either the *atl6-1 mkp1* or *atl31 mkp1* mutants (Figure 4.9). This result, much like the previous *atl6-1 atl31* pathology results, suggests two potential hypotheses. The first is that ATL6 and ATL31 function together in PTI, so removing either one of them from the plant is sufficient to both increase susceptibility and to suppress the enhanced resistance seen in *mkp1* plants. The other is that the limits of this specific system of carrying out pathology are being reached and the plants are infected to saturating levels. A crucial follow up experiment will be to replicate these pathology experiments by leaf infiltration in adult plants.
Figure 4.8: Generation of the *atl6-1 atl31 mkp1* double mutant.

The *atl6-1 atl31 mkp1* triple mutant was confirmed by genotyping plant leaf tissue using primers listed in Table 4.1. *wt*, represents the wild type genes; *mu*, represents the mutated genes with T-DNA insertion; *actin* was used for internal reference. Mutant was generated by Lingyan Jiang.
Figure 4.9: Resistance to *Pst* DC3000 seen in *mkp1* plants is dependent upon ATL6 or ATL31.

12-day-old seedlings were immersed in $2 \times 10^7$ cfu mL$^{-1}$ solution of *Pst* DC3000 and in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ (2 seedlings per sample) before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences using an ANOVA P<0.0001.
A \textit{cbl} quintuple mutant phenocopies the \textit{atl} knock outs

The \textit{atl6} or \textit{atl31} mutants suppress the enhanced resistance in \textit{mkp1} mutants. The only other mutant known to have this effect is \textit{mpk6}, the MAP kinase regulated by MKP1. Therefore, as both MPK6 and ATL6/ATL31 appear to function within the MKP1-regulated pathway, a plausible possibility is that activation of MPK6 results in phosphorylation, and perhaps enhanced activity, of the ATLs. However, based on the known phosphorylation sites that change during flg22 responses, ATL6 and/or ATL31 cannot be directly phosphorylated by a MAPK. MAPKs are all proline directed kinase, and no proline is found in the +1 position of the ATL phosphorylation sites. Based on the minimal R-X-X-S phosphorylation motif, in these proteins, possible kinases that may be involved include CDPKs, SnRKs, or CBL/CIPKs. Previous work investigating the role of ATL31 in carbon/nitrogen ratio response showed that this protein can be phosphorylated \textit{in vitro} by CIPK14 working with CBL8 (Yasuda \textit{et al.}, 2017). Therefore, CIPKs appear to be possible candidates for the kinase acting between MPK6 and ATL6/ATL31.

Because CBL-CIPK family members are often somewhat functionally redundant, we began to investigate this possibility using a \textit{cbl1/4/5/9/10} (hereby denoted \textit{cbl}) quintuple mutant from Sheng Luan’s lab (UC Berkley), which effectively eliminates all CBL-CIPK activity that would function at the plasma membrane. When Col-0 and \textit{cbl} plants were infected with \textit{Pst} DC3000, bacteria grew to similar levels in Col-0 and \textit{cbl} plants (Figure 3.10A left). To test more directly for possible effects on PTI in the mutant, \textit{Pst} DC3000\textit{ΔavrPto/avrPtoB} was
used. As expected, Col-0 plants showed lower levels of infection than when using the fully virulent pathogen, indicating an effective PTI response. However, similar to the results seen in the atl6 or atl31 mutants, cbl plants showed increased bacterial levels, comparable to those from the wild-type pathogen (Figure 4.10A right). Therefore, the cbl plants are also inhibited in their ability to mount a PTI-associated defense response. I also infected plants with Pst DC3000 (hrcC−), a strain of Pst that does not have a functioning T3SS. When infected with Pst DC3000 (hrcC−), Col-0 plants showed significantly less bacterial growth compared to the virulent strain. In comparison, significantly more growth of Pst DC3000 (hrcC−) was observed in cbl plants, indicating a hampered ability to mount an effective PTI response (Figure 4.10B). Because the cbl mutants phenocopy the atl plants, it suggests they are potentially functioning linearly in the same pathway, indicating that the CBL-CIPK network could be a missing piece in this pathway.
Figure 4.10: cbl quintuple knockout plants phenocopy atl6 or atl31 plants in that they are unable to mount an effective PTI response.

(A and B) 12-day-old seedlings were immersed in $2 \times 10^7$ cfu mL$^{-1}$ solution of Pst DC3000 and in a 24-well plate. Three days post infection (3 dpi), seedlings were removed, rinsed in sterile H$_2$O, and ground in 10 mM MgCl$_2$ (2 seedlings per sample) before serial dilution plating with 6 samples per experiment. Values are mean ± SEM (n=18) pooled from 3 independent experiments. Letter groups indicate significant differences using an ANOVA P<0.0001.
cbl plants are unaffected in flg22-induced growth inhibition

Because cbl plants seem to be mimicking atl plants in a defense response, I next wanted to determine if they behave similarly during other PTI-associated responses. Col-0 and cbl seedlings were transferred from MS plates to liquid MS media containing varying amounts of flg22 to assay for PAMP-induced growth inhibition. At both 100 nM and 10 μM flg22, cbl and Col-0 plants showed very similar reductions in fresh weight, indicating that growth inhibition in response to flg22 is not altered in cbl mutants (Figure 4.11).
Figure 4.11: flg22-induced growth inhibition is unaltered in cbl quintuple mutants.

6-day-old plants were placed in 0.5x liquid MS media in 24-well plates (one seedling per well) along with varying concentrations of flg22. After 12 days of elicitation, fresh weight was measured for each individual seedling. Values are means ± SEM (n=54) pooled from 3 independent experiments. Letter groups indicate significant differences calculated from a two-way ANOVA, P<0.05.
Future Directions

This work identifies ATL31 as a novel component in PTI-associated defense and places it downstream of the MKP1-dependent pathway. In addition, it indicates that ATL31 functions either redundantly or in tandem with ATL6, laying the groundwork for several future lines of experimentation. Moving forward, a key question to be addressed is the relationship between ATL6 and ATL31. As indicated previously, using adult plants, and trying other pathology experiments, such as spray inoculation or infiltration of atl6-1 atl31 double mutants, should begin to clarify this. If the proteins are functioning somewhat redundantly, I would expect to see increased susceptibility in the double mutant compared to the singles. If they are functioning together, I would expect removal of one to be enough to get the full susceptibility phenotype. This result should help to identify the relationship between ATL6 and ATL31 and help determine next steps moving forward.

This work also puts forward the CIPK-CBL network as having a potentially crucial role in PTI, specifically in the same pathway as ATL6 and ATL31. An immediate next step already underway in the lab, is to generate a sextuple mutant with the cbl quintuple knockout and mkp1. This will allow us to determine if the CBLs are indeed functioning in the same pathway, allowing us to place them more firmly as functioning linearly with ATL6/31. Other future experiments include further dissecting which CIPKs/CBLs are required using lower order mutants as well as in vitro kinase assays using the CIPKs with ATL6/31 as the substrate to
confirm a functional interaction between these proteins. Based on the work carried out so far, the groundwork is set to quickly determine more members of this pathway moving forward and better understand how exactly PTI functions.

Materials and Methods

**Plant Material and Growth Conditions**

*Arabidopsis* seeds were sterilized using a 10% bleach and 0.05% Tween-20 solution for 15 minutes at room temperature while spinning before being thoroughly washed with sterile H$_2$O. Seeds were then plated onto 0.5% agar containing 2.1 g L$^{-1}$ Murashige and Skoog (MS) salts (PhytoTechnology Laboratories) pH 5.7, 1% sucrose and 6.4 g mL$^{-1}$ MS salts vitamin powder (PhytoTechnology Laboratories). Plates were then vernalized at 4°C for at least two days before being moved into the Percival and grown with a 11/13-hour day/night cycle at 22°C. These conditions were also used during seedling infection and PAMP elicitation. The atl31-1 (GK-746D08), and mkp1 mutants were all in the Columbia (Col-0) background and have been described previously (Bartels *et al.*, 2009; Sato *et al.*, 2009). The atl6-1 atl31 double mutant and atl6-1 atl31 mkp1 triple mutant were previously generated in the lab by Lingyan Jiang (see thesis for details). The atl31 mkp1 double mutant was generated and confirmed by PCR genotyping. *cbl1/4/5/9/10* quintuple knock out mutants were obtained from Sheng Luan at UC-Berkeley and have not yet been described.
Seedling *Psuedomonas* Growth Measurements

At least four days prior to infection, a glycerol stock stored at -80°C of *Psuedomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), *Pst* DC3000 (hrcC), *Pst* DC3000ΔavrPto/avrPtoB, or *Pst* DC3000 (AvrRpm1) was streaked onto King’s B (KMB) agar plates containing 30 µg mL⁻¹ rifampicin (*Pst* DC3000 or *Pst* DC3000 (hrcC)) or 30 µg mL⁻¹ rifampicin and 50 µg mL⁻¹ kanamycin (*Pst* DC3000ΔavrPto/avrPtoB or *Pst* DC3000 (AvrRpm1)) and incubated for two days at room temperature. A master plate of bacteria was stored at 4°C, and a working plate was streaked two days before infection and grown at room temperature. One day prior to infection, 12-day-old seedlings were aseptically transferred from MS agar plates to 1 mL sterile H₂O in a single well of a 24-well microtiter plate (two seedlings per well). After transferring seedlings, microtiter plates were returned to growth chamber at 21°C overnight for 16-20 hours. For pretreatment experiments, experiments began with 12-day-old seedlings and after initial equilibration overnight, sterile H₂O was replaced with 1 µM flg22 for 8 hours before infection.

Immediately prior to infection, bacterial inoculums were prepared by scraping bacteria off of KMB agar plates and resuspending in sterile H₂O for a final concentration of 2×10⁷ cfu mL⁻¹ (OD₆₀₀=0.02). H₂O or flg22 in microtiter plates was then replaced with the bacterial solution and plates were returned to the growth chamber until sample harvesting. To quantify colony forming units (cfu), serial
dilution plating was used three days post-infection. Two seedlings of each genotype were briefly rinsed with sterile H₂O before homogenization in 400 µL of sterile 10 mM MgCl₂. 10-fold dilutions were then prepared with 10 µL being spotted on KMB agar plates containing necessary antibiotics. After two days, colonies were counted, and cfus were calculated.

**Spray Infection Pseudomonas Growth Measurements**

After sterilization, *Arabidopsis* seeds were sown directly onto soil, vernalized at 4°C for two days, and grown in a 10/14-day/night cycle at 21°C for 2-3 weeks. Immediately prior to infection, bacterial inocula of *Pst* DC3000 and *Pst* DC3000 (AvrRpm1) were prepared as described above to a final concentration of 2×10⁸ (OD₆₀₀ = 0.2). Three dpi, seedlings were removed from soil, briefly rinsed in sterile H₂O, followed by cfu calculation as described above.

**Seedling Growth Inhibition by flg22**

6-day-old MS plate-grown seedlings were rinsed and then transferred into 24-well plates (1 seedling per well) containing 1 mL of liquid MS medium (2.1 g L⁻¹ MS salts, pH 5.7, and 1% sucrose) with or without varying concentrations of flg22 (100 nM, 1 µM, or 10 µM). After 12 days, seedlings were rinsed in sterile H₂O, patted dry, and weighed individually.
Table 4.1: Sequences of primers used for PCR genotyping of T-DNA insertion mutants.

<table>
<thead>
<tr>
<th></th>
<th>ATL6-1 FP</th>
<th>ATL6-1 RP</th>
<th>LBb1.3</th>
<th>WT – ATL6-1 FP and ATL6-1 RP</th>
<th>mut – ATL6-1 FP and LBb1.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>atl6-1</strong> (SALK_083652)</td>
<td>CTTCATGGGCTTCTTCTCTATCT</td>
<td>CCGGTAGCACCTAACCTAATC</td>
<td>ATTTTGCCGATTTCGGAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>atl31</strong> (GK-746D08)</td>
<td>GAGGAGAGTGAAACGCAA</td>
<td>AGATCGGTTTCCACCACCAC</td>
<td>ATATTGACCATCATACTCATTGC</td>
<td>WT – ATL31 FP and ATL31 RP</td>
<td>mut – ATL31 RP and 08409 LB</td>
</tr>
<tr>
<td><strong>mkp1</strong></td>
<td>ACAAGTCTATGGAAGAAG</td>
<td>TGTCTTTCCACCACGCATC</td>
<td>ACGCAGCAGATACGCTGG</td>
<td>WT – MKP1 FP and MKP1 RP</td>
<td>mut – MKP1 RP and T-DNA RB</td>
</tr>
</tbody>
</table>
CHAPTER 5

Conclusions and Future Directions

Plants have evolved complex mechanisms to defend themselves against bacterial pathogens. When a plant encounters a bacterium, it uses plasma-membrane (PM)-localized proteins known as pattern recognition receptors (PRRs) to perceive the presence of non-self molecules, or pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) (Felix et al., 1999; Zipfel et al., 2006). After perception, the plant cell initiates a set of responses with the goal of restricting pathogen growth, collectively known as pattern-triggered immunity (PTI). These responses include MAP kinase activation, ROS production, and defense-related transcript accumulation (Boller and Felix, 2009; Zipfel et al., 2004; Lacombe et al., 2010). Simultaneously, the bacteria recognize the presence of a potential host and induce their type-III secretion system (T3SS) to inject effector proteins into the plant cell. These effectors interfere with defense responses in the plant and facilitate nutrient acquisition (Collmer et al., 2009; Hogenhout et al., 2009). One of the major goals of the plant during a PTI response is to restrict effector delivery to better avoid infection (Crabill et al., 2010; Oh et al., 2010). The plant does this by restricting the secretion of T3SS-inducing metabolites into the extracellular space (Anderson et al., 2014). With fewer metabolites in the apoplast, the bacteria are hampered in their ability to induce their T3SS and successfully inject effector
proteins. A robust PTI response restricts pathogen growth, increasing the plant’s likelihood of survival.

If the bacteria are able to successfully inject effector proteins into the plant cell, they inhibit defense responses in the plant, making infection easier (Collmer et al., 2009; Hogenhout et al., 2009). However, plants have evolved another layer of defense, known as effector-triggered immunity (ETI), that is activated when a specific effector is recognized by its cognate R protein (Cui et al., 2015; Jones and Dangl, 2006). During ETI, the plant responds more robustly compared to PTI, often resulting in cell death, to stave off more widespread infection.

Previous work in the lab established MKP1 as a negative regulator of PTI (Anderson et al., 2011). mkp1 knockout plants show increased resistance against bacterial infection as well as decreased effector delivery (Anderson et al., 2014). T3SS-inducing bioactive metabolites are decreased in the extracellular space of mkp1 plants; and adding back these metabolites suppresses both the resistance and effector delivery restriction in mkp1 plants. In addition, adding back the same T3SS-inducing metabolites to PAMP-treated plants also suppresses PTI-associated resistance and effector delivery restriction, indicating that mkp1 plants mimic PTI-treated plants, making them a useful tool to better understanding PTI. Before the work presented in this dissertation, the only known member of the MKP1-dependent pathway was MPK6, the direct substrate of MKP1 (Anderson et al., 2011; Anderson et al., 2014).
E3 ligases play several different roles in PTI in *Arabidopsis thaliana* (Zhou and Zeng, 2017). However, they have been shown to exclusively function early in PTI, modulating initial signaling events and resulting in alterations to canonical PTI responses such as ROS production and defense-related transcript accumulation (Lu et al., 2011; Trujillo et al., 2008; Stegmann et al., 2012; Lee et al., 2020; Ma et al., 2020). The PM-localized E3 Ligase *Arabidopsis Tóxicos en Levadura 6* (ATL6) was shown in our lab to be differentially phosphorylated after PAMP elicitation (Nühse et al., 2007), indicating a potential role in PTI. Due to its proposed function and localization to the PM, in addition to its differential phosphorylation, ATL6 was a strong candidate for functioning in PTI.

In chapter 2, I firmly establish ATL6 as a key positive regulator of PTI. In knockout mutants, *atl6* plants are unable to manifest a robust PTI response, leading to susceptibility compared to wild type after PAMP elicitation or infection with compromised strains of *Pst* DC3000. I also show that ATL6 is required for full PTI-associated effector delivery restriction, as bacteria show increased effector delivery in *atl6* mutants after PAMP-elicitation compared to wild type plants. Interestingly, while *atl6* plants are clearly impaired in their ability to mount a PTI response, several canonical PTI-associated phenotypes are unaltered: including ligand-induced PRR degradation, ROS production, and defense-related transcript accumulation. These results set ATL6 apart from other E3 ligases functioning in PTI because it does not appear to be involved in these responses, suggesting it plays a role further downstream in a PTI response.
Due to its role as a regulator of effector delivery restriction, we hypothesized that ATL6 may play a role in the MKP1-dependent pathway. To investigate, a double knockout (atl6-1 mkp1) double mutant was generated and used for experimentation. When infected with Pst DC3000, atl6-1 mkp1 plants show wild type levels of infection, suppressing the resistance normally seen in mkp1 plants. This result indicates that the resistance in mkp1 plants is dependent upon ATL6, linking these two proteins in a pathway. However, not all mkp1-associated phenotypes are dependent on ATL6. mkp1 mutants show increased ROS production and hyperaccumulation of a subset of defense-related transcripts compared to wild type. In the atl6-1 mkp1 double mutants, these phenotypes were maintained, indicating they are independent of ATL6. These data suggest that ATL6 plays a more specific role in regulating effector delivery restriction, but not other molecular PTI-associated responses. A probable hypothesis is that ATL6 targets key metabolite transporters for degradation to remove them from the PM. If ATL6 targets transporters responsible for secretion of T3SS-inducing metabolites, loss of ATL6 from the plant would result in the continued presence of these transporters and, therefore, metabolites in the apoplast, leading to increased susceptibility. A key follow-up experiment will be to perform effector delivery assays on atl6-1 mkp1 double mutants. If the loss of ATL6 can suppress the effector delivery restriction seen in mkp1 plants, it further suggests that ATL6 plays a specific role in this pathway, limiting secretion of T3SS-inducing metabolites.
Further experiments will aim to pin down the role of ATL6 in PTI more specifically, and to precisely identify its mode of action. A key question left in this pathway is to identify the potential transporters required for modulating the extracellular levels of T3SS-inducing metabolites. A proteomics study has already been carried out in the lab using elicited and naïve plants, so mining that data could provide insight into potential candidates. In addition, using the protocol already established, performing a PM proteomics experiment using both naïve and PAMP-elicited wild type and atl6 mutant plants could identify candidates. Proteins that accumulated in elicited atl6 plants, but not wild type would be of particular interest. Profiling the metabolome of naïve and elicited atl6 and atl6-1 mkp1 exudate vs. that of wild type would also further probe whether ATL6 regulates the accumulation of these T3SS-inducing metabolites. Based on the pathology results, I would expect PAMP-elicited atl6-1 and naïve atl6-1 mkp1 double mutant plants to suppress the decreased accumulation of T3SS-inducing metabolites seen in mkp1 plants.

Another line of experimentation will specifically look at the dynamics of ATL6 in the cell during a defense response. Although ATL6 is differentially phosphorylated during a PTI response, it remains unclear if phosphorylation is important for ATL6 function. Using phospho-null and phospho-mimic constructs I generated for complementation assays, simple pathology experiments should quickly determine if phosphorylation is important for ATL6’s role in PTI. If the phospho-null mutant can suppress the susceptibility of the knockout, it would
suggest that the presence of the protein is enough to complement the phenotype, and that phosphorylation is not required for ATL6 activity. If the opposite is true, it would support the idea that PTI-induced phosphorylation is required for its role. I also have started to optimize an ATL6 antibody which can be utilized to study ATL6 accumulation and localization. Preliminary key experiments will include looking at ATL6 accumulation after both PAMP elicitation and Pst DC3000 infection. Additionally, using differential centrifugation for PM enrichment, we can better determine the localization of ATL6 after various treatments. These experiments should provide valuable insight into the dynamics of ATL6 during a defense response.

In chapter 3, I identified MKP1 as a negative regulator of ETI. When infected with ETI-triggering strains of Pst DC3000, mkp1 knockout plants showed increased resistance compared to wild type. This was a particularly surprising result as we had previously thought a potential tradeoff for the removal of MKP1 would be a decreased ability to mount an effective ETI response. Because fewer effector proteins are translocated into mkp1 plants (Anderson et al., 2014), we hypothesized that they would not be able to respond as effectively to ETI-triggering strains. Interestingly, not only does MKP1 function as a key negative regulator of ETI, it appears to be functioning in a distinct pathway to how it functions in PTI. mkp1 plants show increased resistance to Pst DC3000, but this resistance is dependent on MPK6 and ATL6. However, when mkp1 mpk6 and atl6-1 mkp1 plants were infected with ETI-triggering strains, they maintained the
resistance seen in \textit{mkp1} mutants. This indicates that \textit{mkp1}-associated resistance to ETI-triggering strains is independent of MPK6 and ATL6, suggesting MKP1 functions in a different pathway during an ETI response.

Based on known and hypothesized MKP1 interacting partners, I utilized other genetic tools to determine if MKP1 functions with MPK3 or the CDPKs during an ETI response. In both cases, the subsequent mutations did not suppress the resistance seen in \textit{mkp1} plants, indicating that their resistance to ETI-triggering strains is independent of MPK3 and the CDPKs. As of now, we don’t have any other candidates for proteins functioning with MKP1 during an ETI response. A next step is to do a mutant screen of EMS-mutagenized \textit{mkp1} plants, probing for suppression of the \textit{mkp1}-associated resistance. We have started optimizing a high-throughput screen using a luminescent strain of ETI-triggering \textit{Pst} DC3000. Screening potential double mutants for suppression of resistance followed by sequencing of individual lines should help identify potential candidates for functioning with MKP1 during an ETI response.

I also showed that although \textit{mkp1}-associated resistance to \textit{Pst} DC3000 is independent of salicylic acid (SA), it is dependent on SA during infection with \textit{Pst} DC3000 (AvrRpm1). At present, it is not clear where MKP1 might intersect with SA, particularly as PR1, a common marker for SA-mediated signaling, is actually suppressed in \textit{mkp1}. Other SA mutants can be used in conjunction with \textit{mkp1} to investigate the interaction more thoroughly. In addition, pathology experiments using adult plants will provide valuable insight into whether this SA dependence
is present throughout the lifespan of *Arabidopsis*. Together, these experiments should begin to elucidate the role of MKP1 in ETI and start to identify other pathway components.

In chapter 4, I began an investigation into ATL31, the protein most similar to ATL6, to determine if it also plays a role in PTI. I show that much like *atl6* plants, *atl31* knockout plants are compromised in their ability to mount a PTI-associated defense response, without other PTI-associated molecular phenotypes being altered. Additionally, after generating an *atl31 mkp1* double knockout, I showed that resistance in *mkp1* plants to *Pst* DC3000 is also dependent on ATL31. Together, these results suggest that ATL6 and ATL31 play similar roles during a PTI response. Interestingly, an *atl6 atl31* double mutant phenocopies the single knockout mutants during an infection by a compromised strain of *Pst* DC3000 without showing a further increase in susceptibility. This suggests two potential hypotheses: 1) ATL6 and ATL31 function together during a defense response, potentially as a dimer, and therefore both are required to function, or 2) I am currently at saturating levels of bacteria in my pathology experiments and can’t observed a more nuanced difference between the single mutants and the double. A key next step will be to carry out pathology experiments in adult plants to see if I truly have reached saturation levels. If the double mutant is more susceptible than the individual single mutants, it suggests that ATL6 and ATL31 are potentially functioning somewhat redundantly. However, if the double mutant still phenocopies the singles, it would support the hypothesis that ATL6 and
ATL31 function together. Either way, it will provide valuable insight on how to proceed.

I also began an investigation into the CBL-CIPK signaling network and its potential role in PTI. CIPK14 functioning with CBL8 has been shown to phosphorylate ATL31 \textit{in vitro} (Yasuda \textit{et al.}, 2017). Because of this potential connection, I wanted to see if the CBL-CIPK pathway functions in a pathway with ATL6 and ATL31 in a defense response. After obtaining a \textit{cbl} quintuple knockout from Sheng Luan at UC-Berkeley, I performed a preliminary set of pathology experiments. In all cases, the \textit{cbl} quintuple mutant phenocopied \textit{atl6} and \textit{atl31} plants, suggesting that it functions linearly with ATL6/31 during a PTI-associated defense response. A crucial next step will be trying to determine how the CBL-CIPK pathway functions in PTI. One important step will be trying to determine if the CIPKs directly phosphorylate ATL6/31. Using an expression construct I previously generated as a substrate, as well as the ATL6 antibody, \textit{in vitro} kinase assays can be used to determine if the CIPKs phosphorylate the ATLs. Another important facet of this project will be to place the CBLs more firmly in the pathway. The lab is currently working on generating higher order \textit{cbl} mutations with MKP1 knocked out as well. If the removal of the CBLs is sufficient to suppress the resistance seen in \textit{mkp1} plants, that will suggest that they play a key role downstream in this pathway. Together, these experiments should determine clearly whether or not the CBL-CIPK network function in the MKP1-dependent pathway.
In summary, the studies performed in this dissertation have identified two novel components in PTI (ATL6/ATL31) and have placed them in the MKP1-dependent resistance pathway. Additionally, MKP1 has been identified as a negative regulator of ETI, functioning in a previously undescribed pathway. Although many answers have been provided as to the roles of these proteins in plant defense, several lines of experimentation have been opened up that will be passed down to future graduate students in the lab.
Appendix A

Generation of genetic tools for future study of ATL6 and ATL31

This appendix is a brief explanation of the generation of several constructs that will be used to further study ATL6 and ATL31 and can be used for reference.

Introduction

Although this dissertation provides a lot of answers to key questions concerning ATL6 and ATL31 and their roles in PTI, there is still work to be done. Performing pathology experiments in the Col-0 background can often prove difficult due to the presence of the R gene SNC1. Because of SNC1, Col-0 plants have a slight background resistance against Pst DC3000 which makes dissecting small differences between genotypes more difficult. Previously, a double knockout mkp1 snc1 line was generated and showed that resistance seen in mkp1 plants is independent of SNC1. However, the presence of SNC1 in other genotypes complicates pathology experiments. To alleviate this issue, I set out to generate atl6 and atl31 as well as higher order mutations in the Ws background. Ws does not have SNC1 and is therefore a useful model for studying plant-pathogen interactions. In this appendix I explain the generation of constructs to create knockout lines in the Ws background.

As previously discussed, previous work in the lab shows that ATL6 is rapidly phosphorylated after PAMP elicitation (Nühse et al., 2007). However, no
follow up has been done to study the potential importance of phosphorylation to ATL6 activity. Here I show the cloning performed to generate phospho-null and phospho-mimic mutations of ATL6, both single and double, that can be used to probe the importance of ATL6 phosphorylation.

**Generation of CRISPR-Cas9 knockout lines in the Ws background**

After successful completion of several key experiments utilizing atl6 and atl31 mutants in the Col-0 background, it became clear that subsequent studies, particularly quantifying effector delivery and bioassaying plant exudate, would be difficult due to complications arising from the presence of the R-protein SNC1 in Col-0. To circumvent this problem, I decided to utilize CRISPR/Cas9 to generate mutant lines in the Ws background, which is lacking SNC1. Using the egg cell promoter controlled CRISPR/Cas9 protocol developed by Bing Yang’s lab, I was able to generate all the genetic tools necessary to complete this project using one construct (Figure A.1).

Firstly, guide RNA (gRNA) sequences were chosen to target both ATL6 and ATL31. To better ensure targeting, two gRNA sequences were selected for each gene to be cloned into individual pCR8 plasmids. Forward and reverse primers were designed for each gRNA and included BsmBI cut site overhangs that allowed for successful ligation into BsmBI-digested pCR8 vectors (Table A.1). gRNA primers were annealed together before ligating gRNA sequences into their respective BsmBI-digested pCR8 vectors. Reactions were then transformed into
DH5α cells and grown on LB plates containing spectinomycin. DNA was then purified by miniprep, and gRNA presence was confirmed using PCR with the specific forward primer and a general pCR8 reverse primer. Plasmids were then sequenced using Sanger sequencing with the pCR8 reverse primer to confirm successful cloning.

Once all four pCR8 plasmids were confirmed to contain their respective gRNAs, Golden Gate cloning was used to move all gRNAs into the pENTR4-gRNA4-ccdB plasmid (subsequently referred to as the donor vector), using BsaI. After restriction digest and ligation, reactions were transformed into DH5α cells and grown on LB plates containing kanamycin. Plasmid DNA was then purified, and positive clones were verified by PCR using the first gRNA (ATL6-1) forward primer and the last gRNA (ATL31-2) reverse primer. Empty donor vector was utilized as a negative control.

After confirming that all four gRNAs were present in the donor vector, LR Gateway cloning was used to move the entire gRNA cassette into the p1300-AtEC-Cas9-GmUbi-GFP-ccdB plasmid (hereafter referred to as the binary vector). Reactions were transformed into DH5a cells and grown on LB plates containing kanamycin. Because the donor and binary vectors contained the same antibiotic resistance cassette, the purified DNA was run on an agarose gel alongside the donor vector to confirm the proper size. After confirming the correct plasmid, PCR with the first gRNA (ATL6-1) forward primer and the last gRNA (ATL31-2) reverse primer was used to confirm the presence of the gRNA cassette in the
binary vector. Confirmed binary vector was then transformed into GV3101 Agrobacterium cells and grown on LB plates containing kanamycin, hygromycin, and rifampicin. Glycerol stocks were made and stored at -80°C to be used for dip transformations.
Figure A.1: Cloning scheme to generate CRISPR-Cas9 constructs.

This model shows the cloning scheme used to generate the CRISPR-Cas9 construct which can be transformed in Ws and mkp1 plants to generate knockout lines.
Table A.1: Primers used to generate the CRISPR-Cas9 construct

<table>
<thead>
<tr>
<th>gRNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>pCR8 Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATL6-1</td>
<td>ATTGCAGACCAAGCGCTCAACCCGG</td>
<td>AAACGGCGCGTTGACGGGTTGCTCTG</td>
<td>pCR8-U6-26</td>
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<tr>
<td>ATL6-2</td>
<td>GTCGGAGGGTAAAACCCGATTGAC</td>
<td>AAACGGCGCGTTACCTTTCCTCC</td>
<td>pCR8-U3b</td>
</tr>
<tr>
<td>ATL31-1</td>
<td>ATTGCTACTATTATCCGGTCGGGC</td>
<td>AAACGGCGACACGGAATAGTAG</td>
<td>pCR8-U6-1</td>
</tr>
<tr>
<td>ATL31-2</td>
<td>GTCACGAAACCGAAGCTTGGCGCGT</td>
<td>AAACCGCGCGCGTGGCCGTTCTCG</td>
<td>pCR8-U3d-tail</td>
</tr>
</tbody>
</table>

*highlighted sequence is added to gRNA so that it will ligate with BsmBI-digested pCR8 vectors*
**Generation of ATL6 phospho-mutants**

To identify a potential role for the observed PAMP-induced phosphorylation of ATL6, phospho-mimic and phospho-null constructs were generated. During PAMP elicitation, two ATL6 serine residues, 278 and 357, are phosphorylated. Therefore we wanted to mutate both to either aspartate (phospho-mimic) or alanine (phospho-null). A pDONR201 vector containing a cloned ATL6 was obtained from TAIR (accession #: 1008804609) to be used as starting material. Primers were then designed at each site to be mutated (Table A.2). A modified protocol from QuikChange II XL Site-Directed Mutagenesis Kit by Agilent was used. Rolling circle PCR was used to synthesize plasmids containing the desired mutation. In the case of the double phospho-mimic and phospho-null mutants, a previously confirmed mutant was used as starting material. For example, once the S278D mutant was sequence confirmed, it was then used to mutate the 357 site so that in the final product both targets were mutated. All plasmids were Sanger sequenced to confirm successful mutations. Gateway cloning was then utilized to move all constructs into the pMDC32 destination vector.
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S278A</td>
<td>For - ACCGGACGAATAGCTCTTGTTTCTCTTAG Rev - CTAAGAAGAAACCAGAACCTATTTCTCGTGCTCCTGAT</td>
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<tr>
<td>S278D</td>
<td>For - ACCGGACGAATAGCTCTTGTTTCTCTTAG Rev - CTAAGAAGAAACCAGAACCTATTTCTCGTGCTCCTGAT</td>
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<tr>
<td>S356A</td>
<td>For - CTTTTCTTAAACCCGCGGTGTTTTGTGAGGAAC Rev - GTTCCTCCACAAAAACCCGCGGTGTTCTAGAAGAAA</td>
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<tr>
<td>S356D</td>
<td>For - CTTTTCTTAAACCCGCGGTGTTTTGTGAGGAAGCTC Rev - GAGTTTCCTCCACAAAAATCCGCGGTGTTCTAGAAGAAA</td>
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</tbody>
</table>

*highlighted portion indicates mutated amino acid sequence*
Conclusions

The generation of knockouts in the Ws background is still in process. I was able to dip transform both Ws and \textit{mkp1} plants and use GFP fluorescence to identify lines where Cas9 was activated. Several of those lines have been grown and allowed to set seed. A next step for another graduate student will be to screen the next generation for individual mutations in \textit{ATL6} and \textit{ATL31}.

The generation of phospho-mutants of \textit{ATL6} is also still in progress. I transformed all mutant constructs into pMDC32. The next step will be to transform those constructs into \textit{Agrobacterium} to then dip transform into Col-0 and \textit{atl6} plants. From there, simple pathology experiments should be enough to determine the importance of phosphorylation in \textit{ATL6} function.
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VITA

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