REGULATION OF PLANT DEFENSE RESPONSES DOWNSTREAM OF PAMP RECEPTORS

by

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Abstract

The primary layer of plant immunity is pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). In PTI, when pattern recognition receptors (PRRs) recognize PAMPs, a highly regulated, rapid downstream signaling response such as activation of mitogen-activated protein (MAP) kinases, production of ROS and defense gene expression is initiated. My study involves the identification of MAP kinases activated in PTI and regulation of PTI responses by E3 ubiquitin ligases. In *Arabidopsis thaliana*, MPK3, MPK4, and MPK6 are activated upon PAMP treatment. However, previous studies suggest that there are more MAP kinases activated upon PAMP treatment. To identify the additional MAP kinases phosphorylated upon PAMP perception, transgenic plants expressing candidate MPKs with a ZZ-FLAG double tag (approx. 17kD) were generated in Col-0 background. Western blot analysis identified three MAP kinases, MPK1, MPK11 and MPK13 that are phosphorylated upon PAMP treatment.

To identify E3 ligases involved in PTI, E3 ligases whose transcripts are up-regulated upon PAMP treatment were selected for our study. Transgenic lines overexpressing candidate E3 ligases were assayed for deficiencies in PTI. Overexpression of *U6*, one of the selected E3 ligases, lead to severe reduction of flg22-induced reactive oxygen species (ROS) production and increased susceptibility to *Pseudomonas syringae* pv. *tomato* (*P.s.t.*) DC3000 *hrcC*¯. Furthermore, when *U6* was overexpressed in BIK1 (a positive regulator of PTI)-HA transgenic plants, there was a decrease in BIK1-HA protein expression, leading to the hypothesis that BIK1 may be a potential target of U6.

Overall, my thesis contributes to a better understanding of the signaling and negative regulation of PTI. Advancing our knowledge in plant immunity leads to the potential of its use in agriculture and plant protection.

Preface

The work described in this thesis is the culmination of research from May 2012 through November 2014. Below is a list of manuscripts (published or in preparation) that comprise this thesis, and the contribution made by the candidate.

Chapter 1, 1.1.6.5 - Heterotrimeric G proteins was modified from the manuscript:

Nitta, Y., Ding, P., Zhang, Y. (2014). Heterotrimeric G proteins in plant defense against pathogens and ABA signaling. *Environmental and Experimental Botany*. DOI: 10.1016/j.envexpbot.2014.06.011

- The candidate collated, summarized, and analysed information from the literature published on this topic, drafted the manuscript, and created the figures under the supervision of Zhang, Y. The manuscript was carefully revised by Ding, P. and Zhang, Y.

Chapter 2 - Identification of additional MAP kinases activated upon PAMP treatment was modified from the manuscript:

Nitta, Y., Ding, P., Zhang, Y. (2014) Identification of additional MAP kinases activated upon PAMP treatment. *Plant Signaling and Behavior*, in press.

- The candidate conducted most of the experiments, prepared the figures, and drafted the manuscript under the supervision of Zhang, Y. Zhang, Y. chose the MAP kinase candidates to be tested. Zhang, Y. also designed the experiments. Ding, P. aided with the construction of the plasmids for the transgenic plants.

Chapter 3 - Identification and analysis of an E3 ligase involved in the regulation of PAMP-triggered immunity describes an on-going project:

- The candidate conducted most of the experiments and prepared the figures. Zhang, Y. selected the E3 ligase candidates to be screened. Zhang, Y. also designed the experiments. Qiu, J. helped with the screening of the candidate E3 ligases. Transgenic lines overexpressing candidate E3 ligases were generated and provided to us by Tong, M. as a collaboration with the lab of Li, X.

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List of abbreviations

35S CaMV	a very strong constitutive promoter found in Cauliflower mosaic virus (CaMV)
AGB1	ARABIDOPSIS G-PROTEIN β-SUBUNIT1
AGG1/AGG2	ARABIDOPSIS G-PROTEIN γ-SUBUNIT1/ARABIDOPSIS G-PROTEIN γ-SUBUNIT2
AvrPphB	an avirulence protein from *Pseudomonas syringae*
AvrPto	an avirulence protein from *Pseudomonas syringae*
AvrPtoB	an avirulence protein from *Pseudomonas syringae*
BABA	β-aminobutyric acid
BAK1	BRI1-associated kinase1
BIK1	BOTRYTIS-INDUCED KINASE1
BKK1	BAK1-like1
BRI1	BRASSINOSTEROID-INSENSITIVE1
cDNA	complementary DNA
CEBiP	rice chitin elicitor-binding protein
CERK1	CHITIN ELICITOR RECEPTOR KINASE1
CFU	colony-forming units
Col-0	an Arabidopsis ecotype; it is also referred as wild-type in this thesis
DNA	deoxyribonucleic acid
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EFR	EF-Tu receptor
EF-Tu	bacterial translation Elongation Factor Tu
elf18	an N-acetylated peptide comprising the first 18 amino acids of bacterial elongation factor Tu
ERF	ethylene response factor
ERK	extracellular signal-regulated kinase
ETI	effector-triggered immunity
FLAG-ZZ	double epitope protein tags consisting of a single or repeated DYKDDDDK sequence (FLAG) and a sequence synthesized from the B domain of Protein A (ZZ)
flg22	flagellin conserved domain of 22 amino acids that are recognized by FLS2
FLS2	FLAGELLIN-SENSITIVE 2; flagellin receptor
GDP	guanosine diphosphate
GPA1	ARABIDOPSIS G-PROTEIN α-SUBUNIT1
GPCR	G protein-coupled receptor

GTP	guanosine triphosphate
Gα	G-protein α subunit
Gβ	G-protein β subunit
Gγ	G-protein γ subunit
HA	hemagglutinin; an epitope protein tag compose of a single or repeated YPYDVPDYA sequence
HECT	Homologous to the E6-AP Carboxyl Terminus; an 40 kDa catalytic domain found at the C-terminus of HECT-class E3 ubiquitin protein ligases
HopAI1	an avirulence protein from *Pseudomonas syringae*
HopS1	an avirulence protein from *Pseudomonas syringae*
IOS1	IMPARED OOMYCETE SUSCEPTIBLITY1
L*er*	Landsberg *erecta*; an Arabidopsis ecotype
LRR	leucine-rich repeat
LYM	lysin-motif protein
LysM	lysin-motif
MAP kinase/MPK	mitogen-activated protein kinase
MAP2K/MAPKK/MKK	MAP kinase kinase
MAP3K/MAPKKK	MAP kinase kinase kinase
MEKK	MAPK/ERK KINASE KINASE
MS media	Murashige and Skoog media
NADPH	nicotinamide adenine dinucleotide phosphate
NB-LRR	nucleotide binding-leucine rich repeat
NHO1	NONHOST1
OD	optical density
P.s.m. ES4326	*Pseudomonas syringae* pv. *maculicola* ES4326
P.s.t. DC3000	*Pseudomonas syringae* pv *tomato* DC3000
P.s.t. DC3000 *hrcC*$^-$	*Pseudomonas syringae* pv *tomato* DC3000 *hrcC*$^-$; mutated in a component of the type three secretion system (TTSS)
PAMP	pathogen-associated molecular pattern
PBL27	PBS1-like27
PBS1	AVRPPHB SUSCEPTIBLE 1
pCambia1305-FLAG-ZZ	a modified pCambia 1305 vector with FLAG-ZZ tags used for constructing plasmid
PGN	peptidoglycan
pGST1	a modified pGreen vector used for constructing plasmid
pmr4	*powdery mildew resistant4*
PR gene	pathogenesis-related gene
PRR	pattern-recognition receptor
PTI	PAMP-triggered immunity
PUB	PLANT U-BOX proteins

R protein	resistance protein
RBOHD	RESPIRATORY BURST OXIDASE HOMOLOG D
RING	Really Interesting New Gene
RLCK	receptor-like cytoplasmic kinase
RLK	receptor-like kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	salicylic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SUMM2	SUPPRESSOR of *mkk1 mkk2*, 2
TAIR	The Arabidopsis Information Resource
T-DNA	transfer-DNA
TTSS	type-three secretion system
Ws-0	Wassilewskija; an Arabidopsis ecotype

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Chapter 1 Introduction – literature review[1]

Plants have evolved a variety of defense mechanisms to protect themselves from diverse microbial pathogens in the environment. The plant immune system consists of two conceptual layers: pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). In PTI, certain molecular motifs associated with groups of pathogens (PAMPs) are recognized by pattern-recognition receptors (PRRs) on the plasma membrane of plant cells. This recognition triggers signal transduction pathways leading to downstream defense responses including callose deposition, stomatal closure and the production of reactive oxygen species (ROS). However, successful pathogens have evolved effectors that suppress this response to overcome the effects of PTI, possibly as a result of co-evolution. Furthermore, to counteract this, plants have evolved another layer of defense, ETI. In ETI, resistance (R) proteins recognize specific pathogen effector molecules to initiate defense responses. The resistance mediated by R proteins are typically amplified versions of PTI responses, often leading to the hypersensitive response (Jones & Dangl, 2006). My research is focused mainly on the signal transduction and regulation that occurs during the activation of the first layer of defense, PTI.

1.1 PAMP-triggered immunity

1.1.1 Flagellin and FLS2

The locomotive apparatus of mobile bacteria, flagellum, is made up of the protein building block flagellin. Bacterial flagellin was identified as the first general elicitor isolated from bacteria, to induce defense-like responses in suspension-cultured tobacco cells (Felix,

[1] A portion of this chapter has been published. Nitta Y, Ding P, Zhang Y. (2014). *Environmental and Experimental Botany*. DOI: 10.1016/j.envexpbot.2014.06.011

Duran, Volko, & Boller, 1999). The N-terminus of bacterial flagellin is highly conserved and a 22 amino acid sequence spanning this domain was found to be a very strong elicitor. This peptide was named flg22 (Felix et al., 1999). When *Arabidopsis thaliana* is treated with flg22, it elicits callose deposition and *pathogenesis-related* (*PR*) gene expression, suggesting an important role of flg22 in the induction of plant defense (Gómez-Gómez, Felix, & Boller, 1999).

To identify components involved in flagellin perception, a forward genetics screen was carried out to look for mutants insensitive to flg22. The receptor kinase FLAGELLIN-SENSING2 (FLS2) was identified in this screen, suggesting its involvement in flagellin recognition (Gomez-Gomez & Boller, 2000). FLS2 consists of an extracellular leucine-rich repeat (LRR) region, a transmembrane motif and a cytoplasmic kinase domain (Gomez-Gomez & Boller, 2000). In the two *fls2* alleles found, one contains a point mutation in the LRR domain and the other has a point mutation in the kinase domain. The insensitivity to flg22 in the two *fls2* mutants can be complemented with the wild-type *FLS2* gene, suggesting that both the LRR and kinase domains are necessary for flg22 perception (Gómez-Gómez, Bauer, & Boller, 2001). FLS2 was further demonstrated to directly bind to flg22 by immunoprecipitation and affinity cross-linking assays in *Arabidopsis* and tomato cells (Chinchilla, Bauer, Regenass, Boller, & Felix, 2006). To find the exact residues responsible for flg22 perception, a double-Ala scanning mutagenesis was performed on the FLS2 LRR, which revealed that FLS2 LRR residues 9 to 15 are specifically responsible for flg22 perception (Dunning, Sun, Jansen, Helft, & Bent, 2007).

1.1.2 Elongation factor-Tu and EFR

Bacterial translation elongation factor (EF)-Tu was also identified as a PAMP. When suspension cultured *Arabidopsis* cells were challenged with crude extracts from *Escherichia coli* GI826, a strain which lacks the flagellin elicitor, defense responses were still observed, suggesting the existence of a PAMP other than flagellin in the crude bacterial extract. Through fractionation of the crude bacterial extract and testing the fractions for elicitor activity, EF-Tu was identified as an elicitor of defense responses. By testing different fragments of the proteins, it was found that the N terminus of EF-Tu contains the elicitor activity. Further analysis showed that an 18 amino acid peptide from the N-terminus of EF-Tu (elf18) exhibits full elicitor activity (Kunze et al., 2004).

Because plants treated with flagellin and EF-Tu activate a common set of defense responses, it was hypothesized that EF-Tu would be recognised by an RLK similar to FLS2. EF-Tu receptor (EFR) was identified by analysing T-DNA insertion mutants of elf-18 induced RLKs which are unable to perceive EF-Tu. When EFR is transiently expressed in *Nicotiana benthamiana,* which lacks the ability to recognize EF-Tu, the plant gained the ability to respond to EF-Tu (Zipfel et al., 2006), confirming the specificity of EFR in recognizing EF-Tu of bacterial pathogens.

1.1.3 Chitin, peptidoglycan and CERK1

Chitin, a component of the fungal cell wall, is also a heavily studied PAMP. Chitin and its fragments have been shown to induce defense responses in both monocots and dicots (Shibuya & Minami, 2001). In rice, the chitin elicitor-binding protein (CEBiP) was shown to play an important role in the recognition of chitin. CEBiP has two extracellular lysin motif (LysM) domains and a transmembrane domain (Kaku et al., 2006). LysM domains are able

to recognize polysaccharides with *N*-acetylglucosamine residues such as peptidoglycan and chitin. To identify proteins involved in the perception of chitin in *Arabidopsis*, knock-out lines of genes encoding proteins with LysM motifs were screened for loss of ROS accumulation in response to the chitin elicitor. This led to the identification of CERK1. The *cerk1* mutant is unable to respond to the chitin elicitor. CERK1 has a transmembrane domain and was shown to localize to the plasma membrane (Miya et al., 2007). CERK1 was also identified in a similar reverse genetics screen where the knock-out lines of proteins which contain LysM motifs were screened for the lack of induction of defense related genes after chitin treatment (Wan et al., 2008). These studies demonstrated the important role of CERK1 in the perception and signaling of chitin induced responses.

In a separate study, CERK1, along with two LysM domain proteins (LYM1 and LYM3), were demonstrated to be required for the perception of another PAMP, bacterial peptidoglycan (PGN), which is structurally closely related to chitin. Using a reverse genetics approach, LYM1 and LYM3 were characterized as possible PGN receptors because of their localization to the plasma membrane and their structure similarity to rice CEBiP. Although the *lym1* and *lym3* mutants had altered PGN induced gene expression and increased virulence to a bacterial pathogen compared to Col-0, they lack a cytoplasmic domain, suggesting that their signaling requires additional proteins. As *cerk1* mutants showed compromised immunity against bacterial pathogens, CERK1 is required for immunity against bacteria as well as fungi. When tested for PGN-induced gene expression, CERK1 was found to be required for the perception of PGN (Willmann et al., 2011). Therefore, CERK1 has dual functions in the perception of two different PAMPs, chitin and PGN.

1.1.4 PAMP responses

Upon PAMP perception by its corresponding PRR, several PTI responses are induced. They can be used to quantify the magnitude of the defense response. Some of these responses include callose deposition, accumulation of ROS and stomatal closure. As stated previously, flg22 is a peptide of flagellin that can induce defenes-related responses. The *Arabidopsis* ecotype Wassilewskija (Ws-0) is known to be unresponsive to flg22. When Ws-0 and *Landsberg erecta* (*Ler*), an ecotype of *Arabidopsis* that is responsive to flg22, were treated with flg22, high callose deposition was seen only in the treated *Ler* leaves but not in Ws-0 (Gómez-Gómez et al., 1999), suggesting that callose deposition is an inducible response by PAMPs.

Callose deposition is thought to strengthen the cell wall and considered to contribute positively to pathogen resistance. However, the *powdery mildew resistant4* (*pmr4*) mutant, carrying a mutation in a callose synthase gene, is more resistant to pathogens even though it lost all induced callose deposition. The *pmr4* mutant showed small lesions on the leaf suggesting hypersensitive response (HR). Because salicylic acid (SA) is often involved in HR, double mutant analysis between *pmr4* and mutants blocking SA defense signaling was carried out. The double mutant showed restored susceptibility to powdery mildew, suggesting that callose deposition is not only an inducible defense response upon PAMP treatment, it is also critical for negative feedback regulation of the SA signaling pathway (Nishimura et al., 2003).

Accumulation of ROS is another early defense response that can be triggered by PAMP perception. The mutants *fls2* and *efr* do not accumulate ROS when treated with its corresponding peptide flg22 and elf18 respectively. However, they do produce ROS when

treated with the opposite peptide (Zipfel et al., 2006). Thus, the accumulation of ROS can also be used to measure the magnitude of defense responses induced by PAMPs. There are many possible effects of this accumulation of ROS, which include strengthening the cell wall and regulating defense by acting as a defense signal or by working with other signaling molecules (Torres, Jones, & Dangl, 2006).

Stomatal closure is also one of the earliest host responses after perceiving a pathogen or PAMPs. In wild-type *Arabidopsis* plants, stomatal aperture was drastically reduced upon flg22 treatment. However, this reduction was not seen in the *fls2* mutant plants when treated with flg22, suggesting the necessity of PAMP perception in reducing stomatal aperture. The importance of this defense-response by the plant is also supported by the discovery of the coronatine virulence factor that pathogens secrete into the plant to block stomatal closure (Melotto, Underwood, Koczan, Nomura, & He, 2006).

1.1.5 The contribution of PTI in plant defense

Phenotypic analysis of mutants defective in PAMP receptors demonstrated the importance of PTI. When wild-type and *fls2* mutant plants were treated with flg22, defense-related genes and resistance to pathogenic bacteria was induced in wild-type plants, but not in the mutants. Although *fls2* and wild-type plants show similar susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*P.s.t.* DC3000) when they are directly infiltrated into the leaf, *fls2* is more susceptible when the bacteria is sprayed onto the leaves, suggesting that flg22 perception by FLS2 plays a role in the early stages of defense which may consist of the prevention of bacterial invasion in the first place (Zipfel et al., 2004). When EFR is mutated in *Arabidopsis*, plants become more susceptible and prone to transformation by the virulent bacteria *Agrobacterium tumefaciens*, which has high EF-Tu

elicitor activity. This indicates that perception of EF-Tu triggers a defense response which hinders *Agrobacterium* mediated transformation (Zipfel et al., 2006). *cerk1* mutants also show defective defense responses such as lack of induction of defense gene expression and accumulation of ROS after treatment with chitin. The *cerk1* mutant is more susceptible to the fungal pathogen *Alternaria brassicicola* (Miya et al., 2007) as well as the bacterial pathogen *P.s.t.* DC3000, indicating that perception of PAMP signaling by CERK1 is important in both fungal and bacterial defense responses (Willmann et al., 2011).

Increasing evidence that pathogenic effectors often target components of PTI also support the importance of PTI in plant defense. The expression of *NONHOST1* (*NHO1*) is strongly induced by flg22 and a non-host bacterial pathogen in a flagellin dependent manner. *NHO1* encodes a glycerol kinase and is required for the resistance against non-host pathogens. *P.s.t.* DC3000 with a mutation in its type-three secretion system (TTSS) strongly induces *NHO1*, but *P.s.t.* DC3000 can only transiently induce *NHO1*. Expression of a number of *P.s.t.* DC3000 effectors such as, HopAI1, HopS1, and AvrPto supresses flg22-induced *NHO1* expression (X. Li et al., 2005), suggesting that pathogen effectors target signaling components of PTI to suppress defense gene expression.

AvrPtoB, another *P. syringae* effector, suppresses basal defense against pathogens in *Arabidopsis* (de Torres et al., 2006). It was found to ubiquitinate the CERK1 kinase domain *in vitro* and degrades CERK1 *in vivo*. *Arabidopsis* plants expressing *AvrPtoB* were unable to initiate defense-related responses upon chitin treatment (Gimenez-Ibanez et al., 2009).

In addition, AvrPto localizes to the plasma membrane and has kinase-inhibition activity. It interacts with FLS2 and EFR, the PRRs for flagellin and EF-Tu respectively. The interaction correlates with its ability to block PTI in tomato plants (Xiang et al., 2008).

Another effector AvrPphB, was shown to block PTI by cleaving receptor-like cytoplasmic kinases downstream of multiple PRRs such as FLS2 and EFR (J. Zhang et al., 2010). These data support the importance of PTI as the primary layer of defense, as pathogens have evolved effector proteins that specifically target components of PTI to overcome this layer of defense.

1.1.6 Downstream signaling

Formation of receptor complexes and rapid signalling of the extracellular PAMP perception to downstream intracellular signal transduction are also heavily studied subjects in PTI. A number of components were found to function downstream of PAMP receptors, including BRI1-associated kinase1 (BAK1), BOTRYTIS-INDUCED KINASE1 (BIK1), IMPARED OOMYCETE SUSCEPTIBLITY1 (IOS1), PBS1-like27 (PBL27), and heterotrimeric G proteins.

1.1.6.1 BAK1

BRASSINOSTEROID-INSENSITIVE1 (BRI1) is a receptor for the brassinosteroid phytohormone and is involved mainly in plant growth and development. The receptor like kinase (RLK) BAK1 was initially identified in a yeast-two-hybrid screen to search for interactors of BRI1 (Nam & Li, 2002). The *bak1* mutant was later found to exhibit reduction in flagellin and EF-Tu induced responses, suggesting an important role of BAK1 in PAMP signalling (Chinchilla et al., 2007; Heese et al., 2007). When BAK1 is mutated together with its homolog BAK1-like1 (BKK1), the *bak1 bkk1* double mutant displays further diminished defense-related responses in ROS production and defense gene induction (Roux et al., 2011). Upon treatment by flg22, BAK1 rapidly associates with FLS2 *in vivo*, shown by immunoprecipitation assays (Chinchilla et al., 2007; Heese et al., 2007). In *N. benthamiana*,

BAK1 was also shown to be required for the perception of other PAMPs such as a bacterial cold-shock protein, a PAMP that acts as a highly active elicitor of defense responses in tobacco (Felix & Boller, 2003), and an oomycete elicitor. This demonstrates BAK1 as an important receptor kinase that works with many different PRRs in response to PAMPs (Heese et al., 2007).

The association between BAK1 and FLS2 was shown to occur instantaneously (<1second) after PAMP treatment. The kinase activity of BAK1 was shown to be necessary for this interaction. After flg22-induced interaction between FLS2 and BAK1, phosphorylation was observed in both FLS2 and BAK1. Phosphorylation of BAK1 with immunoprecipitated EFR was seen when plants were treated with elf26 (a peptide of EF-Tu), suggesting that BAK1 also forms a complex with EFR. Thus, phosphorylation of PAMP receptors may be a starting point of intracellular signal transduction that occurs after PAMP perception (Schulze et al., 2010).

1.1.6.2 BIK1

BIK1 was originally identified as a factor of plant defense-responses against necrotrophic pathogens (Veronese et al., 2006). BIK1 is rapidly phosphorylated in response to flg22 treatment. It interacts with both BAK1 and FLS2 *in vivo* and *in vitro*. Similar to BAK1, BIK1 is also a common component in multiple PAMP signaling pathways. The *bik1* mutant plant displayed reduced defense-related responses upon different PAMP treatments, and exhibited enhanced susceptibility to bacterial pathogens (Lu et al., 2010; J. Zhang et al., 2010).

In *Arabidopsis*, rapid accumulation of ROS is mediated by the NADPH oxidase, RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD). PAMP treatment induces

the phosphorylation of RBOHD, leading to the production of ROS. Recently it was shown that RBOHD is constitutively associated in a complex that includes the PRRs at the plasma membrane. The N-terminal domain of RBOHD directly binds BIK1 and BIK1 phosphorylates RBOHD upon PAMP treatment. Phosphorylation of RBOHD by BIK1 is required for its function in ROS production (Kadota et al., 2014; L. Li et al., 2014). These data demonstrate the important role of BIK1 in signal transduction immediately downstream of the PAMP receptors to promote the production of ROS.

1.1.6.3 IOS1

IOS1 was recently identified in a reverse genetics analysis of genes whose expression is induced by the non-protein amino acid, β-aminobutyric acid (BABA). IOS1 encodes a malectin-like RLK. The *ios1* mutants showed enhanced susceptibility towards *P. syringae* pathogens. They were also impaired in defense responses such as induction of defense gene expression and callose deposition upon PAMP treatment and bacterial infection. On the other hand, when IOS1 is overexpressed in *Arabidopsis*, the overexpression lines showed enhanced resistance to *P. syringae* compared to wild-type. Interestingly, IOS1 was found to exist in a complex with FLS2 and EFR, demonstrated by *in vitro* pull down assays and co-immunoprecipitation and mass spectrometry analyses. IOS1 also interacts with BAK1 and positively regulates the FLS2/BAK1 complex formation (Chen et al., 2014). It is likely that IOS1 functions as a positive regulator of signal transduction together with or immediately downstream of PRRs.

1.1.6.4 PBL27

PBL27 was identified as the close homologue of the rice receptor like cytoplasmic kinase (RLCK) 185 that was known to directly interact with rice CERK1. CERK1 was found

to phosphorylate PBL27 *in vitro*. The *pbl27* mutant was impaired in chitin-induced defense-responses such as callose deposition and resistance to a fungal pathogen. However, *pbl27* does not show an effect when treated with flg22, suggesting that the signaling mediated by PBL27 was specific to CERK1 (Shinya et al., 2014).

1.1.6.5 Heterotrimeric G proteins

Heterotrimeric G proteins were initially discovered as signal transducers in the mammalian system in the 1970s. They are made up of three distinct subunits, Gα, Gβ and Gγ. In the widely accepted G-protein paradigm in mammals, the three subunits form an inactive heterotrimer with a GDP-bound Gα monomer and Gβγ dimer in the absence of an agonist (activating ligand). The binding of ligands to the G-protein-coupled receptors (GPCRs) results in exchange of GDP from the Gα subunit with the GTP and the release of the G protein complex (Oldham & Hamm, 2008). The GTP-bound Gα subunit and the Gβγ dimer subsequently dissociates from each other and both activate downstream effectors (Hamm, 1998; Klopffleisch et al., 2012).

Treatment with flg22, elf18 or chitin leads to rapid induction of ROS in wild type plants. In *Arabidopsis g-protein β-subunit 1* (*agb1*) mutants, ROS induction by flg22 and chitin is reduced, whereas elf18-induced ROS production is almost completely abolished (Liu et al., 2013). In contrast, mutations in the Gα subunit GPA1 have no effect on the ROS induction by flg22, elf18 or chitin. Analysis of ROS induction in the *Arabidopsis g-protein γ-subunit 1* (*agg1*) *agg2* double mutant showed that flg22 and chitin-induced oxidative burst is also greatly reduced and elf18-induced oxidative burst is nearly abolished. These data suggest that AGB1 and AGG1/2 function downstream of the RLKs FLS2, EFR and CERK1 to regulate PAMP-triggered oxidative burst (Liu et al., 2013).

In wild type plants, pre-treatment with flg22, elf18 or chitin leads to increased resistance to bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*P.s.t.*) DC3000. flg22, elf18 and chitin-induced resistance was greatly reduced in *agb1* and *agg1 agg2* mutants, but not affected in *gpa1* mutant plants (Liu et al., 2013), further supporting that AGB1 and AGG1/2 function downstream of the RLKs FLS2, EFR and CERK1 to regulate PAMP-triggered immune responses.

1.2 MAP kinase signaling

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases. They are involved in many biological processes by responding to a diverse array of stimuli. MAP kinase cascades play important roles in plant immunity. In PTI, generally, stimulated transmembrane receptors such as the *Arabidopsis* PRR, FLS2, activate the MAP kinase kinase kinases (MAP3K/MEKK) which then sequentially phosphorylate the downstream MAP kinase kinases (MAP2K/MKK) which in turn activates MAP kinases (MAPK/MPK). This signaling cascade then leads to defense responses such as the induction of defense-gene expression. Analysis of the *Arabidopsis* genome revealed, 20, 10 and 60, MAPKs, MKKs, and MEKKs' respectively (Asai et al., 2002).

In *Arabidopsis thaliana*, two MAP kinase cascades have been discovered to be activated downstream of PAMP receptors. Upon treatment with flg22, one cascade leads to the activation of MKK4 and MKK5 which then activates MPK3 and MPK6 (Asai et al., 2002). The MEKK phosphorylated upstream of this cascade is still unknown. This cascade positively regulates defense responses as it leads to the transcriptional activation of many defense related gene expressions (Asai et al., 2002). The other cascade leads to the activation of MPK4 through MEKK1 and MKK1/MKK2 (Ichimura, Casais, Peck, Shinozaki, &

Shirasu, 2006; Suarez-Rodriguez et al., 2007). This cascade was demonstrated to negatively regulate a set of defense responses because the loss of function of either MEKK1 or MPK4 shows accumulation of salicylic acid (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007).

In a recent study, a screen was performed to identify suppressors of the *mkk1 mkk2* autoimmune phenotype, which led to the identification of a nucleotide binding leucine-rich repeat (NB-LRR) protein, SUPPRESSOR of *mkk1 mkk2,* 2 (SUMM2) (Z. Zhang et al., 2012). Loss of function of SUMM2 supresses cell death in *mkk1 mkk2* as well as in *mekk1* and *mpk4.* Thus, the MEKK1-MKK1/MKK2-MPK4 cascade negatively regulated SUMM2-mediated immunity. Interestingly, it was also found that this cascade positively regulates basal defense targeted by the pathogenic effector HopAI1 from *P. syringae*. When HOPAI1 inactivates MPK4, SUMM2-mediated defense responses are activated (Z. Zhang et al., 2012). It was also shown that MPK4 specifically negatively regulates MEKK2, a MAP3K functioning upstream of SUMM2 (Kong et al., 2012).

Furthermore, when upstream components such as BAK1, IOS1, and PBL27 are mutated, they show reduced phosphorylation of MAPKs upon treatment with their corresponding PAMP peptide (Chen et al., 2014; Roux et al., 2011; Shinya et al., 2014). These data reveal the signaling cascade that is activated upon PAMP treatment, which eventually lead to defense-related responses such as defense gene induction.

1.3 Negative regulation of PTI by ubiquitination

When plant immunity is constitutively up-regulated, an autoimmune phenotype is seen where plants usually become very small as seen in *mekk1*, *mkk1 mkk2*, and *mpk4* mutant plants. Thus, it is critical to have negative regulators to control the time or duration of the immune responses. Recently ubiquitination has been shown to play an important role in the

negative regulation of PTI. Ubiquitination, often involved in protein degradation, involves three enzymes, the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase enzyme (E3) (Ikeda & Dikic, 2008).

PUB22, PUB23 and PUB24 are three E3 ligases that negatively regulate PTI. All three genes are induced upon flg22 treatment and infection by bacterial and oomycete pathogens. The *pub22 pub23 pub24* triple mutant showed enhanced and prolonged ROS production upon flg22, elf18, and chitin treatment, suggesting that PUB22, PUB23 and PUB24 act to down-regulate ROS production upon PAMP treatment. Other defense responses such as defense gene induction and MAPK activation are also enhanced in the triple mutant, further confirming its negative regulatory role in PTI (Trujillo, Ichimura, Casais, & Shirasu, 2008).

PUB13, another E3 ligase, was identified through a yeast-two-hybrid screen using BAK1 as bait. PUB13 and its close homologue PUB12 were shown to interact with BAK1 *in vivo* as well. Upon flg22 treatment, PUB12 and PUB13 also associate with FLS2, but in *bak1* mutants the interaction between PUB12/PUB13 and FLS2 was lost, indicating the need for BAK1 in this complex formation. Because BAK1 interacts with PUB12 and PUB13 without PAMP induction, it is likely that BAK1 and PUB12/PUB13 are always in a complex and are recruited to BAK1/FLS2 complex upon PAMP perception. PUB12 and PUB13 function as E3 ligases to ubiquitinate FLS2 *in vitro* and are required for degradation of FLS2. In the *pub12* and *pub13* mutant plants, FLS2 accumulates to higher levels. When treated with the flagellin peptide, both mutants produced significantly more ROS compared to wild-type. They also displayed enhanced callose deposition and were more resistant to bacterial

pathogens compared to wild-type. These data suggest that PUB12 and PUB13 are critical negative regulators of FLS2 mediated PTI (Lu et al., 2011).

The focus of my research is on the regulation of signal transduction downstream of PRRs. My thesis is specifically focused on MAPKs that are phosphorylated upon PAMP treatment and an E3 ligase that negatively regulates PTI.

Chapter 2 Identification of additional MAP kinases activated upon PAMP treatment[2]

2.1 Summary

Mitogen-activated protein (MAP) kinase cascades play important roles in plant immunity. Upon pathogen associated molecular pattern (PAMP) treatment, MPK3, MPK6 and MPK4 are quickly activated by upstream MKKs through phosphorylation. Western blot analysis using α-phospho-p44/42-ERK antibody suggests that additional MPKs with similar size as MPK4 are also activated upon PAMP perception. To identify these MAP kinases, seven candidate MPKs with similar sizes as MPK4 were selected for further analysis. Transgenic plants expressing these MPKs with a ZZ-3xFLAG double tag of 17 kD were generated and analyzed by western blot. MPK1, MPK11 and MPK13 were found to be phosphorylated upon treatment with flg22. Our study revealed additional MAPKs being activated during PAMP-triggered immunity.

2.2 Introduction

Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases. They are involved in signal transduction during many biological processes through responding to diverse arrays of stimuli. MAP kinase cascades play critical roles in plant defense against pathogens (Meng & Zhang, 2013). During pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), transmembrane receptors such as FLAGELLIN-SENSING 2 (FLS2) activate MAP kinase kinase kinases (MEKKs), which subsequently phosphorylate downstream MAP kinase kinases (MKKs) that in turn activate MAP kinases (MPKs).

[2] A version of this chapter has been published. Nitta Y, Ding P, Zhang Y. (2014) *Plant Signaling and Behavior*, in press.

In *Arabidopsis*, there are 20 MPKs, 10 MKKs and about 60 putative MEKKs (MAPK-Group, 2002). Two MAP kinase cascades have been shown to be activated downstream of PAMP receptors. One leads to activation of MKK4 and MKK5 and the downstream MPK3 and MPK6 (Asai et al., 2002). The MEKK functioning in this cascade is still unknown. Downstream of the MAP kinases, ethylene response factor 6 (ERF6) was identified as a substrate of MPK3/MPK6 and ERF104 was identified as a substrate of MPK6 (Bethke et al., 2009; Meng et al., 2013). Activation of these ERF proteins is critical for defense against fungal pathogens.

Another cascade downstream of PAMP receptors leads to activation of MPK4 through MEKK1 and MKK1/MKK2 (Gao et al., 2008; Ichimura et al., 2006; Nakagami, Soukupova, Schikora, Zarsky, & Hirt, 2006; Pitzschke, Djamei, Bitton, & Hirt, 2009; Qiu et al., 2008; Suarez-Rodriguez et al., 2007). This cascade negatively regulates defense responses mediated by the NB-LRR resistance protein SUMM2 (Z. Zhang et al., 2012). Inactivation of MPK4 by the bacterial effector protein HopAI1 leads to activation of SUMM2-mediated immune responses. The MEKK1-MKK1/MKK2-MPK4 kinase cascade was also found to positively regulate basal defense, as *summ2 mekk1* and *summ2 mkk1 mkk2* mutant plants exhibit enhanced susceptibility to pathogens (Z. Zhang et al., 2012).

The α-phospho-p44/42-ERK antibody (Cell Signaling Technology, Inc., #4370s) recognizes a conserved phosphorylation motif of MAP kinases. It has been widely used to analyze MAP kinase phosphorylation in animals and plants. In Arabidopsis, three immunoreactive bands are usually detected in a western blot analysis of samples treated with the elicitor flg22 (Bethke et al., 2012; Liu et al., 2013), a peptide derived from bacterial flagellin that is recognized by FLS2 (Gomez-Gomez & Boller, 2000). In the *mpk6* single

mutant, the band of the highest molecular weight is absent, indicating that phosphorylated MPK6 is typically detected as the top band. In *mpk3*, the middle band is absent, indicating that phosphorylated MPK3 is detected as the central band. The intensity of the lower band with the smallest molecular weight is reduced in *mpk4*, but is not affected in the knockout mutant of its close homolog MPK11 (Bethke et al., 2012). In the *mpk4 mpk11* double mutant, the lower band is still present, but its intensity is further reduced compared to that in *mpk4* (Bethke et al., 2012), suggesting that MPK11 is also phosphorylated after flg22 treatment and flg22-treatment activates additional MAP kinases that co-migrates with MPK4 during sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE).

In this study, we sought to identify MAPKs that have similar sizes as MPK4 and exhibit phosphorylation upon flg22 treatment.

2.3 Materials and methods

2.3.1 Construction of plasmids

Constructs expressing MPK1, MPK2, MPK5 MPK7, MPK11, MPK12, and MPK13 with a FLAG-ZZ fusion tag driven by their native promoters were created for the MAP kinase activation assay. Individual MAP kinase fragments with restriction enzyme sites on each end were amplified using the primers shown in table 1. These fragments were digested and ligated into a pCambia 1305 modified vector with a FLAG-ZZ epitope tag.

Table 1 Primers used for the construction of plasmids

Primer Name	Sequence
MPK1-BamHI-F	CGC**GGATCC**TTCGTGGTTGTCTAATCCAC
MPK1-XhoI-R	CCGCCG**CTCGAG**GAGCTCAGTGTTTAAGGTTG
MPK2-BamHI-F	CGC**GGATCC**CATGGGTTTAACTGTTCTTG
MPK2-PstI-R	AAAA**CTGCAG**AAACTCAGAGACCTCATTGT
MPK5-KpnI-F	CGG**GGTACC**CATGGATAATGTTGGAGATCHA
MPK5-XhoI-R	CCGCCG**CTCGAG**AATGCTCGGCAGAGGATTGA
MPK7-KpnI-F	CGG**GGTACC**CATCCTTAGATGTTACCACT
MPK7-PstI-R	AAAA**CTGCAG**GGCATTTGAGATTTCAGCTT
MPK11-BamHI-F	CGC**GGATCC**ATGGGTTGATGTCTATCTTGA
MPK11-XhoI-R	CCGCCG**CTCGAG**AGGGTTAAACTTGACTGATTC
MPK12-KpnI-F	CGG**GGTACC**TTGGAAATCCCTTTCGGATG
MPK12-BamHI-R	CGC**GGATCC**GTGGTCAGGATTGAATTTGA
MPK13-BamHI-F	CGC**GGATCC**TTGGCAATATGCAATTGGAGG
MPK13-XhoI-R	CCGCCG**CTCGAG**CATATTCTTGAAGTGTAAAGAC

2.3.2 MAP kinase protein activation assay

Each epitope-tagged MAP kinase construct was transformed into Col-0 plants. T2 plants which expressed the MPK-FLAG-ZZ fusion protein, detected by the α-FLAG (sigma) antibody, were chosen for the MAP kinase activation assay. Twelve-day old transgenic plant seedlings grown on Murashige and Skoog (MS) medium, untreated or treated with 1μM flg22 for 10 minutes were collected. The tissue was frozen, grinded and finally boiled in sodium dodecyl sulfate (SDS) buffer. Western blot analysis was performed on the samples. Phosphorylated MAP kinases were detected using the α-p44/42-ERK antibody which is specific for recognizing phosphorylated MAP kinases.

2.3.3 Pathogen growth assay using *P.s.t.* DC3000 *hrcC*$^-$

Growth of *P.s.t.* DC3000 *hrcC*$^-$ was measured in *mpk1*, *mpk11*, and *mpk13* using Col-0 as a control. Plants were grown under short-day conditions (12-h day/12-h night cycles). Leaves of four-week-old plants were inoculated with *P.s.t.* DC3000 *hrcC*$^-$ (OD_{600} = 0.002).

Two leaves per plant were infiltrated and one leaf disc from each infiltrated leaf was collected. The two leaf discs from the two infiltrated leaves from the same plant were combined to form one sample. These samples were grinded, diluted, and plated on LB plates to count the colonies and ultimately calculate the colony-forming units.

2.4 Results and discussion

MAP kinase candidates were chosen based on their protein sizes. Prior to phosphorylation, MPK4 is 42.9 kD. Seven MAP kinases whose protein sizes are between 42.2kD-43.2kD and that are expressed in leaf tissue based on the microarray database at The Arabidopsis Information Resource, specifically MPK1, MPK2, MPK5, MPK7, MPK11, MPK12 and MPK13, were selected for further studies.

To test whether these candidate MAP kinases are phosphorylated upon treatment with flg22, we analyzed the knockout mutants of the predicted MAP kinases by western blot using the α-phospho-p44/42-ERK antibody, and did not observe any consistent difference between the wild type and the single mutants. Most likely there is redundancy between the MAP kinases that masks the phenotype in single mutants. We then took an alternative approach to detect single MAP kinase phosphorylation upon flg22 treatment using transgenic plants expressing epitope tagged candidate MAP kinases. The epitope used is a FLAG-ZZ double tag. The FLAG tag is approximately 1kD in size, while the ZZ tag is approximately 16kD, which was synthesized from the B domain of Protein A (Forler et al., 2003). By fusing the MAP kinase to the double tag, the protein size is expected to be increased by approximately 17kD, allowing detection of the phosphorylated individual candidate MAP kinases apart from the endogenous proteins.

Constructs expressing MPK1, MPK2, MPK5, MPK7, MPK11, MPK12 and MPK13 with a C-terminal FLAG-ZZ tag under their own promoters were generated and transformed in Col-0 wild type plants. Transgenic lines expressing the fusion proteins were identified by Western blot using an α-FLAG antibody and used for subsequent phosphorylation analysis. As shown in figure 1, treatment with flg22 results in strong increases in phosphorylated MPK1-FLAG-ZZ, MPK11-FLAG-ZZ and MPK13-FLAG-ZZ detected by the α-phospho-p44/42-ERK antibody, suggesting that MPK1, MPK11 and MPK13 are phosphorylated upon flg22 induction.

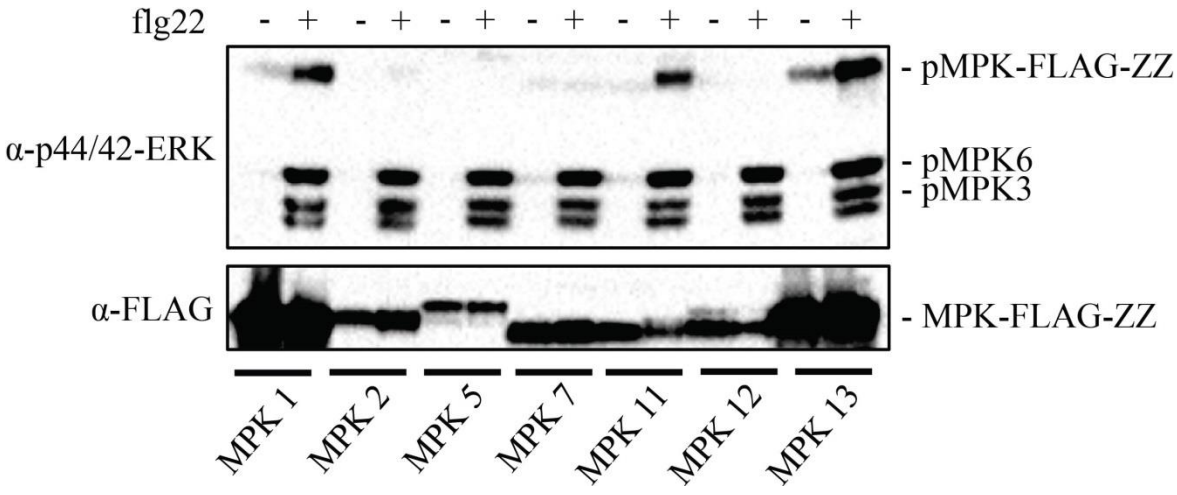

Figure 1 Activation of MPK-FLAG-ZZ fusion proteins in transgenic plants by flg22. 12-day-old seedlings grown on ½ MS medium were treated with or without 1μM flg22. Samples were taken 10 min after treatment. The MPK-FLAG-ZZ fusion proteins were detected using an α-FLAG antibody (Sigma). Phosphorylated MAP kinases were detected using the α-p44/42-ERK antibody.

To test whether the identified MAP kinases are important for PTI, the single mutants of *mpk1*, *mpk11* and *mpk13* were assayed for growth of the non-pathogenic bacteria *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC*$^-$ (*P.s.t.* DC3000 *hrcC*$^-$). As shown in figure 2, the MAPK single mutants showed no enhanced susceptibility to *P.s.t.* DC3000 *hrcC*$^-$, suggesting that loss of individual MAP kinases does not affect PAMP-triggered immunity against *P.s.t.* DC3000 *hrcC*$^-$.

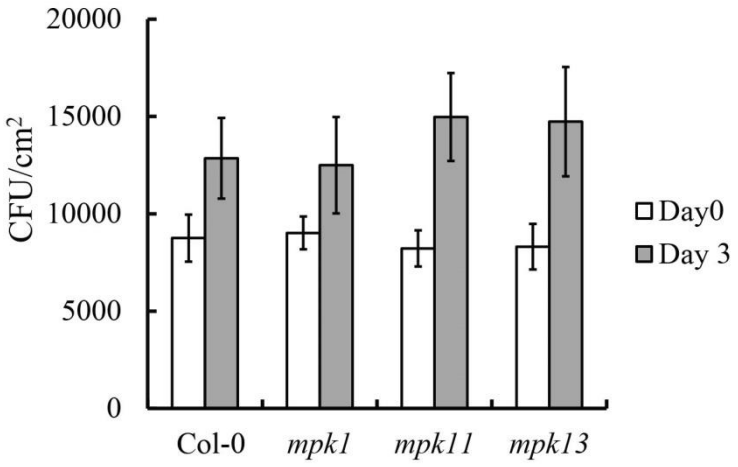

Figure 2 Growth of *P. s. t.* DC3000 *hrcC*$^-$ in Col-0, *mpk1*, *mpk11* and *mpk13* plants.
Leaves of five-week-old plants were inoculated with *P. s.t.* DC3000 *hrcC*$^-$ (OD_{600} = 0.002). Leaf discs in the inoculated area were collected to measure bacterial titers at days 0 and 3. Plants were grown under short-day conditions (12-h day/12-h night cycles). Error bars represent standard deviation scores from means of six samples.

In summary, we have identified additional MAP kinases that are activated in response to flg22 treatment. Lack of obvious defects in PTI against *P.s.t.* DC3000 *hrcC*$^-$ suggests potential functional redundancy among these MAPKs. Analysis of combined mutants of the MAPKs may be required to elucidate their roles in plant defense against pathogens. Future identification of the target proteins of these MAPKs and their upstream MEKKs and MKKs are also critical in understanding how they function in plant immunity.

Chapter 3 Identification and analysis of an E3 ligase involved in the regulation of PAMP-triggered immunity

3.1 Summary

Protein ubiquitination is a common modification for the regulation of different pathways and events in eukaryotic organisms. E3 ubiquitin ligases are key factors in determining substrate specificity of ubiquitination, which most often leads to protein degradation. To identify E3 ligases involved in PTI, E3 ligases whose transcripts are up-regulated upon PAMP treatment were selected as candidates. Transgenic lines overexpressing these candidate E3 ligases were assayed for deficiencies in PTI by quantification of reactive oxygen species (ROS) produced upon flg22 treatment and growth of the non-pathogenic bacteria, *Pseudomonas syringae* pv. tomato (*P.s.t.*) DC3000 *hrcC*⁻. Primary screens showed that overexpression of U6, one of the candidate E3 ligases, leads to severe reduction of flg22-induced ROS production and increased susceptibility to *P.s.t.* DC3000 *hrcC*⁻. Further analysis showed that ROS production was also reduced in the U6 overexpression plants when induced by elf18 and chitin. These results suggest that U6 plays an important role in negatively regulating PTI. As overexpression of U6 causes reduction in the accumulation of the BIK1-HA fusion protein in transgenic plants, we hypothesize that U6 may target BIK1, a positive regulator downstream of a number of PAMP receptors, for ubiquitination and further degradation. Future in-depth analysis of the biochemical and genetic interactions between U6 and BIK1 will reveal their biological relationships.

3.2 Introduction

Ubiquitin is a small conserved protein that is able to covalently modify and often form chains on its target proteins. This modification is called ubiquitination and is often marking its target proteins for degradation. Ubiquitination is regulated by the ubiquitin-26S proteasome system which involves sequential activities of three enzymes, an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2), and an ubiquitin ligase (E3). E3s are key factors in determining substrate specificity (Ikeda & Dikic, 2008). They can be divided into three main groups depending on their conserved domains; HECT, RING-type/U-box, and Cullin-based (Cheng & Li, 2012).

The ubiquitin-26S proteasome pathway is an extremely important and intricate regulatory mechanism in plants. In *Arabidopsis* over 5% of its proteome is predicted to be involved in this pathway (Smalle & Vierstra, 2004). In recent years, E3 ligases, PUB12, PUB13, PUB22, PUB23, and PUB24 have been identified to negatively regulate PTI (Lu et al., 2011; Trujillo et al., 2008), suggesting that E3 ligases may be important regulators in plant immunity. However, even with these discoveries there is still a great deal that is not known about the regulation of PTI. Therefore I hypothesized that there are additional E3 ligases that are involved in the regulation of PTI.

To explore this hypothesis, a reverse genetics screen was carried out on E3 ligases whose transcripts are up-regulated upon PAMP treatment. Transgenic plants overexpressing the candidate E3 ligases were used to screen for defects in PTI. Two PTI assays were used to screen the transgenic plants; a bacterial growth assay using *P.s.t.* DC3000 *hrcC*$^{-}$ and PAMP-induced production of ROS assay. *P.s.t.* DC3000 *hrcC*$^{-}$ is a non-pathogenic bacterium that is often used for testing PTI because it has a mutation in a component of the type three

secretion system (TTSS). With a defective TTSS, *P.s.t.* DC3000 *hrcC*$^-$ is not able to secrete effectors into the plant, allowing us to see the effects of the bacterial PAMPs alone. ROS was also used for the screen because the accumulation of ROS upon PAMP treatment can also be used as a method to measure the magnitude of defense responses.

In this chapter, I will describe the identification of *U6* as a regulator of PTI. *U6* overexpression plants were more susceptible to *P.s.t.* DC3000 *hrcC*$^-$ and produced less ROS when induced by PAMPs compared to the Col-0 wild-type control. *U6* transcript levels are also induced upon flg22 treatment and bacterial infection. This information suggests that U6 plays an important role in PTI. A possible target of U6 was also investigated to further reveal the function of U6.

1.3 Materials and methods

1.3.1 Screening candidate E3 ligases

23 E3 ligases were expressed in the Col-0 ecotype using a Cauliflower Mosaic Virus (CaMV) 35S promoter with an N-terminus HA tag. Seeds of the transgenic plants overexpressing the candidate E3 ligases were kindly provided to us by Dr. Xin Li's lab. These transgenic plants were specifically tested for altered growth of bacterial pathogen and production of ROS.

1.3.2 Pathogen growth assay using *P.s.t.* DC3000 *hrcC*$^-$

Growth of *P.s.t.* DC3000 *hrcC*$^-$ was measured in all the overexpression lines of candidate E3 ligases using Col-0 as a control. Plants were grown under short-day conditions (12-h day/12-h night cycles). Leaves of four-week-old plants were inoculated with *P.s.t.* DC3000 *hrcC*$^-$ (OD_{600} = 0.002). Two leaves per plant were infiltrated and one leaf disc from each infiltrated leaf was collected. The two leaf discs from the two infiltrated leaves from the

same plant were combined to form one sample. These samples were grinded, diluted, and plated on LB plates to count the colonies and ultimately calculate the colony-forming units (CFU).

1.3.3 PAMP-induced ROS production assay

The flg22-induced accumulation of ROS was quantified in all transgenic lines overexpressing candidate E3 ligases using Col-0 as a control. Plants were grown under short-day conditions (12-h day/12-h night cycles). Leaves of four-week-old plants were cut into slices and placed in water in 96-well plates. The plates were incubated for approximately 12 hours at room temperature. After incubation, water was removed and 1μM flg22 elicitor solution with 20μM luminol and 10μg mL^{-1} horseradish peroxidase was added to each sample. Luminescence was recorded using a 96 microplate luminometer (Infinity M200 Pro). elf18 (1μM) and chitin (200μg mL^{-1}) induced ROS production was quantified using the same method.

1.3.4 *U6* transcript expression analysis

For *U6* transcript expression analysis, RNA was extracted from twelve-day old Col-0 seedlings grown on Murashige and Skoog (MS) medium, untreated and treated with 1μM flg22 for 2 hours. The RNA was reverse-transcribed to obtain the complimentary DNA (cDNA) for real-time PCR analysis to determine the expression level of *U6* using the primers U6-RT-F and U6-RT-R, shown in table 2. RNA was also extracted from soil-grown four-week-old Col-0 plants inoculated with *Pseudomonas syringae* pv. *maculicola* ES4326 (*P.s.m.* ES4326) (OD_{600} = 0.001) at 0, 12 and 24 hour time points. The cDNA obtained from this extracted RNA was also used for real-time PCR analysis to determine the expression level of *U6* using the same primers.

For *U6* transcript expression analysis in Col-0, *U6* OX-1 and *U6* OX-7, RNA was extracted from twelve-day-old seedlings grown on Murashige and Skoog (MS) medium. The RNA was reverse-transcribed to obtain the cDNA. Real-time PCR analysis was used to determine the expression level of *U6* using the primers U6-RT-F and U6-RT-R, shown in table 2.

Table 2 Primers used for real-time PCR of *U6*

Primer Name	Sequence
U6-RT-F	AGCAGCAATACTAGCCGGAG
U6-RT-R	AGAGCCTTTGATTAAACTCC

1.3.5 Construction of plasmids

Constructs carrying the dominant-negative forms of *U6* were produced to create overexpression of *U6* dominant-negative transgenic plants. Site-directed mutagenesis was performed by overlapping PCR of the region using 35S-HA-U6 in a modified pGreen vector (provided by Dr. Xin Li's lab) that was originally used to create the overexpression transgenic plants of *U6* as template. Two fragments including the mutated nucleotide were amplified by PCR using pGreenST1-F/U6-HtoY-R and U6-HtoY-F/pGreenST1-R (table 3). Then the two overlapping fragments were mixed together and used as template to amplify the full length gene using the two outside primers (pGreenST1-F and pGreenST1-R). The final PCR product was then cloned into pGST1 with a CaMV 35S promoter and N-terminus HA tag (35S-HA-U6-H110Y). Another dominant negative form of *U6* was created by the same methodology except using two fragments amplified by the primers pGreenST1-F/U6-CtoS-R and U6-CtoS-F/pGreenST1-R (table 3) instead (35S-HA-U6-C116S).

BIK1 driven by the CaMV 35S promoter with a FLAG-ZZ fusion tag was also created for interaction analysis. A BIK1 fragment with KpnI and BamHI restriction enzyme sites was amplified using the primers, BIK1-KpnI-F and BIK1-BamHI-R shown in table 3. This fragment was digested and ligated into a pCambia1305 modified vector with a FLAG-ZZ epitope tag.

Table 3 Primers used for the construction of plasmids

Primer Name	Sequence
pGreenST1-F	GAAGACGTTCCAACCACGTC
pGreenST1-R	GACGCCTATGATCGCATGAT
U6-HtoY-F	CTGACGAATTGTCGATATATATTTCATCGG
U6-HtoY-R	CCGATGAAATATATATCGACAATTCGTCAG
U6-CtoS-F	ATTTCATCGGGGATCTTTGGACCGTTGGAT
U6-CtoS-R	ATCCAACGGTCCAAAGATCCCCGATGAAAT
BIK1-KpnI-F	TAGAGTCGACGGTACCATGGGTTCTTGCTTCAGTTC
BIK1-BamHI-R	CGCGGATCCCACAAGGTGCCTGCCAAAAG

1.3.6 BIK1 protein expression assay

35S-HA-U6 (provided by Dr. Xin Li's lab) and the dominant-negative constructs, 35S-HA-U6-H110Y and 35S-HA-U6-C116S were transformed into BIK1-HA transgenic plants (J. Zhang et al., 2010). Leaves from four-week-old plants were collected from the T2 transgenic plants. The tissue was frozen, grinded and finally boiled in sodium dodecyl sulfate (SDS) buffer. Western blot analysis was performed on the samples using α-HA antibody.

1.3.7 Co-immunoprecipitation in tobacco

To test the interaction of BIK1 and U6, *Agrobacterium* carrying 35S-BIK1-FLAG-ZZ and 35S-HA-U6-C116S constructs were co-inoculated into tobacco. The transiently transformed tissue was collected after 48 hours and protein was extracted. The extracted protein was immunoprecipitated with an agarose-conjugated α-FLAG antibody (Sigma).

Western blot analysis was performed on the input and elution with the α-FLAG (Sigma) and α-HA antibody (Roche).

1.4 Results and discussion

Based on The Arabidopsis Information Resource (TAIR) microarray database, 23 E3 ligases whose transcripts are up-regulated upon PAMP treatment were chosen to test for their involvement in PTI (Table 4). Six independent transgenic lines per construct were used to screen for increased growth of *P.s.t.* DC3000 *hrcC*$^-$ and deficiencies in the production of ROS. Preliminary results from the screen suggest that overexpressing U6, a RING-type/U-box E3 ligase, lead to deficiencies in PTI (Table 4).

U6 was initially chosen as a candidate because its transcript levels were induced upon PAMP treatments. To confirm this, flg22 was used to activate PTI. As shown in figure 3A, a significant increase in *U6* transcription level was detected two hours after flg22 treatment. To test whether *U6* transcript is also up-regulated by bacterial infection, *P.s.m.* ES4326 was inoculated into Col-0 and transcript levels were analyzed at 0 hours, 12 hours and 24 hours. As shown in figure 3B, 24 hours after bacterial infection, *U6* transcript was highly induced. This indicates that both PAMP treatment and bacterial infection lead to up-regulation of *U6* expression.

Table 4 Scored results of the growth of *P.s.t.* DC3000 *hrcC*$^-$ and production of ROS in the overexpression lines of candidate E3 ligases

Code Name	*P.s.t.* DC3000 *hrcC*$^-$ growth	ROS production
U1	No difference	No difference
U4	No difference	No difference
U6	++	--
U8	No difference	No difference
U9	No difference	No difference
U10	No difference	No difference
U11	No difference	-
U13	No difference	-
U15	No difference	No difference
U17	No difference	No difference
U18	No difference	No difference
U19	No difference	-
U20	+	No difference
U21	No difference	No difference
U26	+	No difference
U28	+	No difference
U30	No difference	No difference
U33	+	No difference
U34	No difference	No difference
U35	No difference	No difference
U36	+	No difference
U37	No difference	No difference
U38	+	No difference
	"+" is less than two times, "++" is more than two times increase in pathogen growth compared to Col-0	"-" is less than two times, "--" is more than two times decrease in production of ROS compared to Col-0

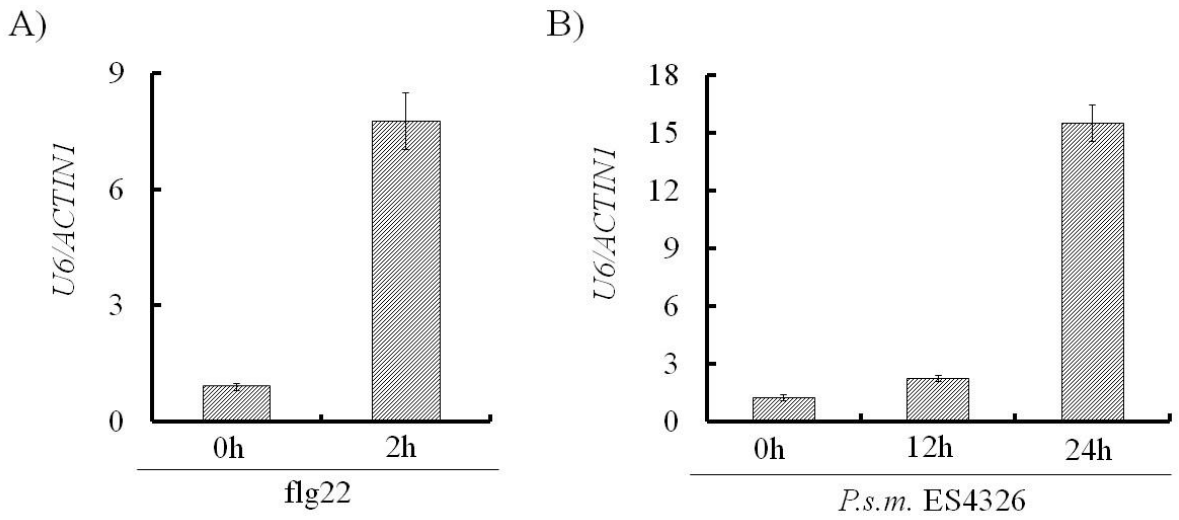

Figure 3 Induction of *U6* expression by flg22 and *P.s.m.* ES4326.

(A) Two-week old Col-0 seedlings were treated with flg22 (1μM). Samples were collected at 0 hours and 2 hours. *U6* transcript levels were analyzed by quantitative real-time PCR.

(B) Four-week old Col-0 plants were inoculated with *P.s.m.* ES4326 (OD_{600} = 0.001). Samples were collected at 0 hours, 12 hours, and 24 hours. *U6* transcript levels were analyzed by quantitative real-time PCR.

Actin 1 was used as the control for both (A) and (B).

The results of the screen were confirmed by quantifying the growth of *P.s.t.* DC3000 *hrcC*$^-$ and production of ROS in two independent lines overexpressing *U6*. The non-pathogenic bacteria, *P.s.t.* DC3000 *hrcC*$^-$ had significantly higher growth in *U6* OX-1 and *U6* OX-7 (figure 4). As shown in figure 5A, the flg22-induced production of ROS was greatly reduced in the same transgenc lines. To determine whether or not the effect seen on

the production of ROS was flg22/FLS2 specific, the same assay was carried out using elf18 and chitin as elicitors. Reduced production of ROS was also observed when elf18 or chitin was used as the inducer (figure 5B & 5C). These data suggest that reduction of ROS accumulation in U6 overexpression lines was not specific to the elicitors, but rather a defect in the general PAMP response.

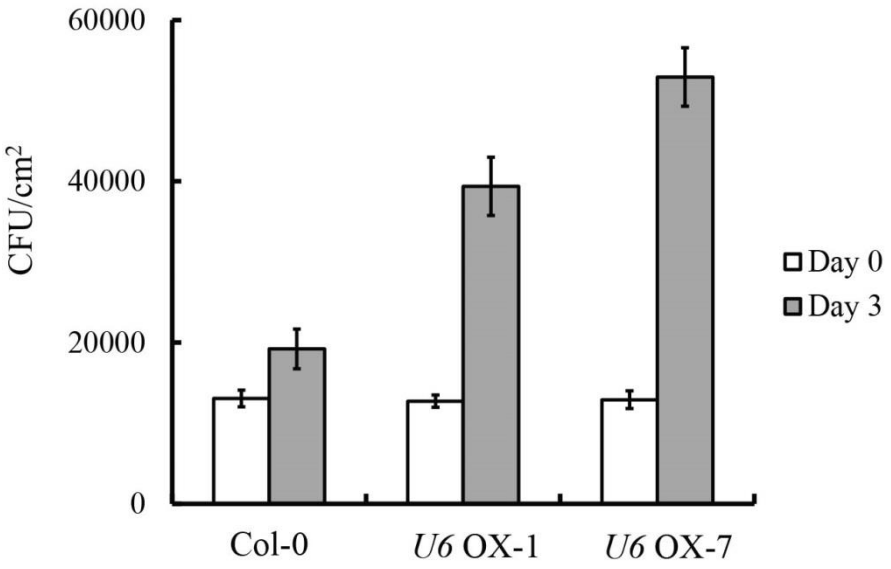

Figure 4 Growth of *P.s.t.* DC3000 *hrcC*⁻ in Col-0 and two independent lines overexpressing U6.

Leaves of four-week old plants were inoculated with *P.s.t.* DC3000 *hrcC*⁻ (OD_{600} = 0.002). Leaf discs in the inoculated area were collected to measure bacterial titers at days 0 and 3. Plants were grown under short-day conditions (12-h day/12-h night cycles). Error bars represent standard deviation scores from means of six samples.

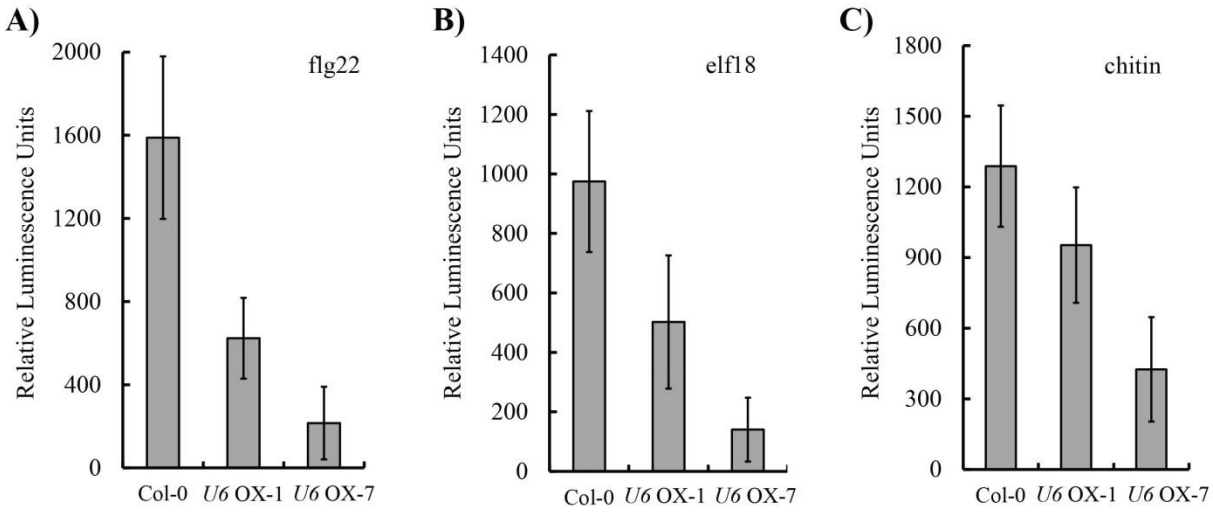

Figure 5 Production of PAMP-induced ROS in Col-0 and two independent lines overexpressing U6.

Oxidative burst triggered by flg22 (A), elf18 (B), or chitin (C). Leaf slices of four-week-old plants were treated with 1μM flg22, 1μM elf18, or 200μgmL^{-1} chitin, and ROS was subsequently measured. Error bars represent standard deviation scores from means of eight samples.

To analyze the degree of overexpression of *U6*, real-time PCR was carried out to determine the transcript levels of *U6* in Col-0, *U6* OX-1 and *U6* OX-7. As shown in figure 6, *U6* transcript levels were much higher in the two transgenic lines overexpressing *U6*. The expression of *U6* in *U6* OX-7 is higher than in *U6* OX-1, which is consistent with higher *P.s.t.* DC3000 *hrcC*$^{-}$ growth and less PAMP-induced production of ROS in *U6* OX-7 compared to *U6* OX-1.

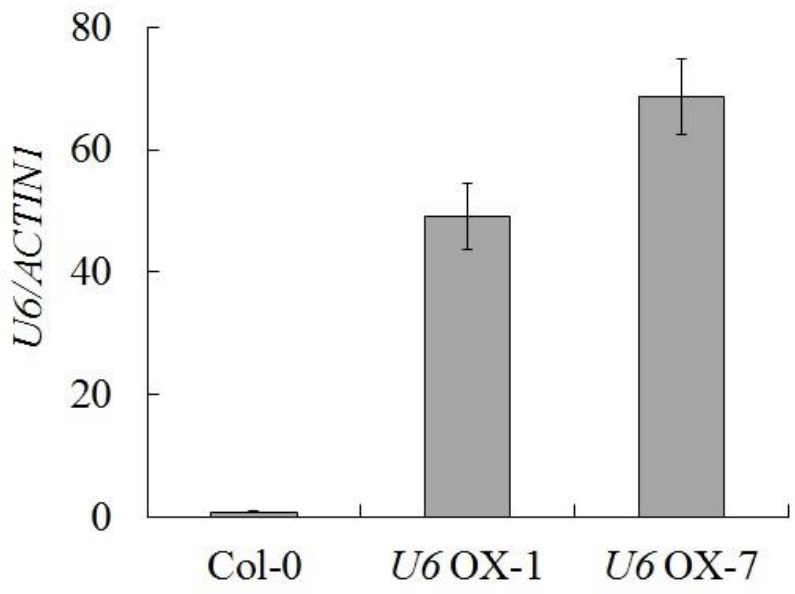

Figure 6 *U6* expression in Col-0, *U6* OX-1 and *U6* OX-7.
U6 transcript levels in two-week-old seedlings were analyzed by quantitative real-time PCR. *Actin 1* was used as the control.

The up-regulation of *U6* transcript following flg22 and *P.s.m.* ES4326 treatment, increase in growth of the non-pathogenic *P.s.t.* DC3000 *hrcC*⁻ and the redued production of PAMP-induced ROS in the *U6* overexpression transgenic plants suggest that U6 is a key negative regulator of early PAMP responses. Because U6 is an E3 ligase, identifying the protein that U6 targets became the focus of my study.

Based on evidence from previous studies I chose BIK1 as a potential target for U6. BIK1 was chosen as a candidate target for U6 because studies showed that BIK1 is a common regulator downstream of PAMP receptors (Lu et al., 2010; J. Zhang et al., 2010). It was also shown to activate RbohD, an NADPH oxidase involved in ROS production (Kadota et al., 2014; L. Li et al., 2014). Furthermore, the *bik1* single mutant shows enhanced susceptibility to *P.s.t.* DC3000 *hrcC*⁻ and also produces less ROS upon PAMP treatment (J.

Zhang et al., 2010). The similarity between the *bik1* phenotypes and the U6 overexpression phenotypes suggests that BIK1 could be a target for ubiquitination by U6.

To test the hypothesis that BIK1 is a target of U6, the 35S-HA-U6 construct (provided by Dr. Xin Li's lab) was transformed into a transgenic line expressing the BIK1-HA fusion protein driven by its native promoter (J. Zhang et al., 2010). 24 independent BIK1-HA and HA-U6 double transgenic plants were planted in the T2 generation to identify single insertion lines. Four lines carrying a single copy of the transgene (T2#7, T2#15, T2#16 and T2#19) were tested for BIK1-HA accumulation using western blot analysis. As shown in figure 7, in all four independent T2 lines overexpressing *U6*, the expression of BIK1-HA was drastically reduced compared to the BIK1-HA parent control. These results provide evidence that overexpressing *U6* has a negative effect on the protein level of BIK1-HA, which supports the hypothesis that BIK1 may be a target of U6.

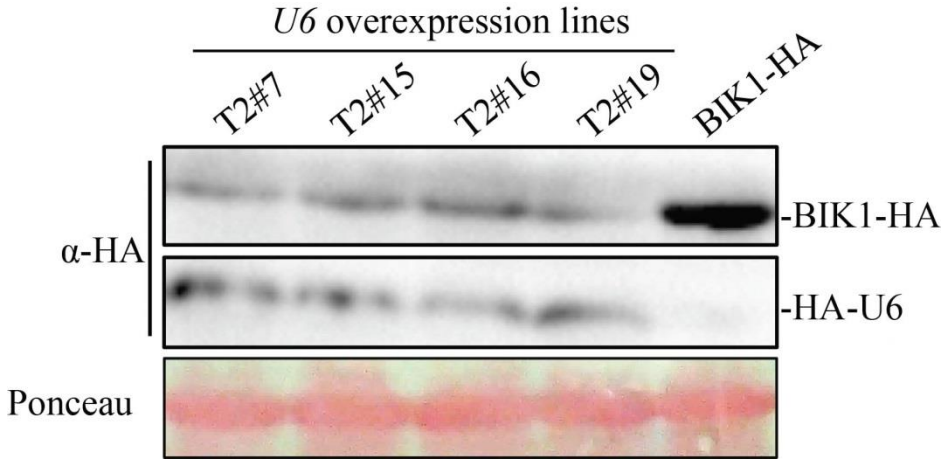

Figure 7 BIK1-HA protein levels in transgenic plants overexpressing *U6*.
Protein was extracted from leaves of four-week-old BIK1-HA transgenic plants and four independent T2 lines of the BIK1-HA and 35S-HA-U6 double transgenic plant. BIK1-HA and U6-HA expression levels were detected via western blot analysis using α-HA antibody. The ponceau staining serves as the loading control.

Next I tested whether the loss of function of U6 affects the accumulation of BIK1. Because U6 has two homologues that may function redundantly with U6, I created *U6* dominant-negative constructs by mutating an amino acid in the zinc binding motif of U6. U6 is in the RING/U-box superfamily and carries a RING finger domain. When essential amino acids in this domain are mutated, the resulting protein has a dominant-negative effect on the endogenous protein, because the mutant form of U6 loses its ability to bind the E2 conjugating enzyme while maintaining its ability to bind to the substrate, thus preventing the substrate from being ubiquitinated (González-Lamothe et al., 2006). The RING finger domain has a consensus sequence where the zinc binds. If one of these amino acids is mutated to destroy the zinc binding structure, it will cause a dominant-negative effect. U6

has a C3H2C3 type RING domain. Figure 8 shows the amino acid sequence of U6 with the C3H2C3 highlighted. Two dominant-negative constructs were created; one by mutating the H to Y and another by mutating the C to S, as highlighted in red in figure 8. The *U6* dominant-negative constructs were subsequently transformed into the BIK1-HA transgenic plants. When the expression levels of BIK1-HA were analyzed in the BIK1-HA and U6 dominant-negative double transgenic plants, BIK1-HA levels showed a significant increase (Figure 9). As the dominant-negative form of U6 affects the accumulation of BIK1-HA in an opposite manner compared to *U6* overexpression, these results further support that U6 regulates the accumulation of BIK1.

```
  1 MGFPVGYSEL LLPKIFFYLL SFLGLIRKLI STMFKIIGLP DFLEPEPVST
 51 SWPDPPPTLT KPDSAAILAG EMLPVVRFSD INRPESECCA VCLYDFENDD
101 EIRRLTNCRH IFHRGCLDRW MMGYNQMTCP LCRTQFIPDH LQLEFNQRLW
151 SQSSAVSSQL LDESSS
```

Figure 8 Amino acid sequence of U6.
The highlighted amino acids are the conserved residues for the C3H2C3 RING motifs. The two amino acids highlighted in red are the amino acids mutated to make the dominant negative forms of *U6*. In one form of the dominant negative *U6*, the H was mutated to a Y and in the other the C to an S.

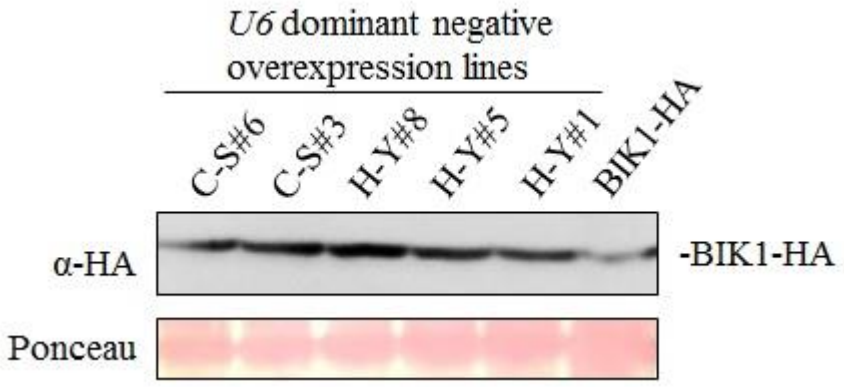

Figure 9 BIK1-HA protein expression in transgenic plants overexpressing dominant negative forms of *U6*.

Protein was extracted from two-week-old seedlings in BIK1-HA transgenic plants and five independent T2 lines overexpressing dominant negative *U6* in the BIK1-HA transgenic background. H-Y#1, H-Y#5 and H-Y#8 are lines expressing *U6* with H_{110} mutated to Y. C-S#3 and C-S#6 are lines expressing *U6* with C_{116} mutated to S. BIK1-HA expression levels were detected via western blot analysis using α-HA antibody. The ponceau staining serves as the loading control.

Analysis of transgenic plants overexpressing *U6* and dominant negative forms of *U6* indicate that U6 affects the protein level of BIK1-HA. To test if U6 and BIK1 interact with each other, co-immunoprecipitation experiments were conducted in tobacco. The 35S-BIK1-FLAG-ZZ and the dominant-negative form of *U6* (35S-HA-U6-C116S) were transiently expressed in tobacco together. The dominant-negative form of the U6 construct was chosen for this assay to prevent potential degradation of BIK1 by U6. Tobacco leaves transfected with 35S-HA-U6-C116S alone (control) or both 35S-BIK1-FLAG-ZZ and 35S-HA-U6-C116S were collected for protein extraction. The protein extracts were then immunoprecipitated with an agarose-conjugated α-FLAG antibody and co-

immunoprecipitation of HA-U6-C116S was then analyzed by western blot using α-HA antibody. Unfortunately, my results showed that the dominant-negative form of U6 protein was enriched greatly in both the negative control and the experimental samples. This suggests that 35S-HA-U6-C116S is able to bind to the α-FLAG antibody or the agarose beads alone. To overcome this problem, HA-U6-C116S will be immunoprecipitated using the agarose-conjugated α-HA antibody. Co-immunoprecipitation of BIK1-FLAG-ZZ will be analyzed by the α-FLAG antibody in future experiments.

Through the screen of E3 ligases that are up-regulated upon PAMP treatment, I found that overexpression of *U6* results in severe deficiencies in PTI. Transgenic plants overexpressing *U6* are more susceptible to *P.s.t.* DC3000 *hrcC*$^-$ and produce less ROS upon PAMP treatment. *U6* transcript was also induced upon flg22 and *P.s.m.* ES4326 treatment. These results suggest an important role of U6 in PTI. As overexpression of *U6* leads to the reduced accumulation of BIK1 and overexpression of dominant-negative forms of *U6* results in increased levels of BIK1 protein, BIK1 may be a target protein of U6. Therefore, the results presented here suggest that U6 negatively regulates PTI by participating in the degradation of BIK1, a key positive regulator downstream of multiple PAMP receptors in PTI. In the preliminary co-immunoprecipitation experiments in tobacco, I could not detect direct interaction of BIK1-FLAG-ZZ and the dominant-negative form of U6 because of technical problems. Further experimentations must be carried out to test whether BIK1 interacts with U6.

Chapter 4 Future directions and conclusions

Microbial pathogens are responsible for losses of crop and forestry industries annually. Current control methods for most pathogens still rely heavily on chemical pesticides, which are environmentally unfriendly and often lead to even more virulent strains of pesticide-resistant pathogens. Increasing efforts seek to discover alternative control methods that are long-lasting and environmentally safe. Better understanding of plant immunity may lead to development of new strategies to engineer plants with enhanced pathogen resistance. My work focuses on two important aspects of the first layer of plant immunity, signaling and regulation of PTI.

By western blot analysis of transgenic plants expressing FLAG-ZZ-tagged candidate MAP kinases, I found that MPK1, MPK11 and MPK13 are phosphorylated upon PAMP treatment. Combined with previous studies, a total of six MAP kinases including MPK1, MPK3, MPK4, MPK6, MPK11 and MPK13 are now known to be activated upon PAMP treatment. When the *mpk1*, *mpk11*, and *mpk13* single mutants were analyzed, I was unable to detect a difference in bacterial growth compared to Col-0. This suggests that loss of individual MAP kinases does not affect PTI and there may be functional redundancy among the MAP kinases.

Future directions for this project are to make combined MAP kinase mutants and test them for phenotypes in PTI deficiency. By studying different combinations of double and possibly triple mutants, it may be possible to identify which MAP kinases function redundantly in PTI.

It is also important to find the upstream components of the identified MAP kinase. Studies using yeast two-hybrid screen (Lee, Huh, Bhargava, & Ellis, 2008) and protein

microarray (Popescu et al., 2009) have previously been carried out to study interactions between all MKKs and MAP kinases in *Arabidopsis*. MKK3 was shown to interact with MPK1 in both studies (Lee et al., 2008; Popescu et al., 2009). MKK1, MKK2 and MKK6 interacts with MPK11, whereas MKK2 and MKK6 interact with MPK13 according to data from the yeast-two-hybrid screen (Lee et al., 2008). MKK1 and MKK2 have already been shown to function upstream of MPK4 (Gao et al., 2008; Qiu et al., 2008). MPK11 and MPK13 are in the same clade as MPK4 (MAPK-Group, 2002), therefore, they may be affected by the same upstream components as MPK4. The MPK1-FLAG-ZZ, MPK11-FLAG-ZZ and MPK13-FLAG-ZZ constructs could be transformed into the knock-out mutants of the possible upstream components (*mkk3, mkk1, mkk2* or *mkk6*) to test if activation of the MAP kinase fusion proteins is affected by the absence of the MKKs.

It is also important to identify downstream targets of the identified MAP kinases. The yeast two-hybrid screen can be carried out to identify candidate target proteins that interact with MPK1, MPK11 and MPK13. These candidates can then be tested using an *in vitro* phosphorylation assay to determine whether the candidate can be directly phosphorylated by MPK1, MPK11 or MPK13.

Through a reverse genetics screen of E3 ligases up-regulated upon PAMP treatment, U6 was identified as a negative regulator of PTI. Plants overexpressing *U6* produced less ROS upon PAMP treatment and were more susceptible to the non-pathogenic bacteria, *P.s.t.* DC3000 *hrcC*$^{-}$. When *U6* was overexpressed in BIK1-HA transgenic plants, the accumulation of BIK1-HA was compromised, suggesting that BIK1 may be a potential target for U6. However, due to technical problems, analysis of the direct interaction of BIK1 and U6 were not successful in tobacco.

The future direction for this project is to test the direct interaction of BIK1 and U6 in *Arabidopsis*. In a separate study, I found that U6 can bind IgG conjugated agarose beads, which is usually used to purify proteins with the ZZ epitope tag. Therefore, BIK1-HA transgenic plants expressing dominant-negative forms of *U6* (also carrying an HA tag) can be used to preform co-immunoprecipitation. Protein extracts from the double transgenic plants can be immunoprecipitated with the IgG beads and co-immunoprecipitation of BIK1-HA can then be analyzed by western blot using α-HA antibody to test *in vivo* interaction between BIK1 and U6.

To confirm the role of U6 in negative regulation of BIK1 accumulation, it is critical to study the loss-of-function mutant phenotypes of U6 and its close homologues. T-DNA insertion lines of U6 and its two homologues can be tested for resistance to the bacterial pathogen (*P.s.t.* DC3000) and production of ROS. Accumulation of BIK1 can also be tested to determine whether there is increased accumulation of BIK1 as seen in the transgenic lines overexpressing the dominant-negative form of *U6*.

This study on U6 provided strong evidence that ubiquitination plays an important role in the regulation of PTI. I showed that the protein level of BIK1, one critical signaling component downstream of PAMP receptors is regulated by the E3 ligase, U6. The identification of U6 also suggests that at least some of the E3 ligases whose transcripts are up-regulated upon PAMP treatment may be important in the regulation of PTI. Therefore, it may be beneficial to test additional E3 ligases that are induced by PAMP treatment or other biotic stresses such as pathogen infection for roles in PTI regulation.

Overall, my work led to the discovery of MAP kinases activated upon PAMP treatment and the identification of the E3 ligase, U6, which is involved in the negative

regulation of PTI. Since overexpression of the dominant-negative form of *U6* in *Arabidopsis* leads to increased accumulation of BIK1, it would be interesting to test these transgenic lines for pathogen resistance. If they show enhanced resistance to pathogens, U6 may be useful in engineering resistant crops. Dominant-negative forms of U6 homologues in crop plants can be created to generate transgenic plants with enhanced resistance to pathogen. Although more careful studies need to be carried out before the application of these findings, understanding the regulation of PTI is an important first step to achieve this goal.

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