Genetic Analysis of ANP2/3-MKK6-MPK4 Cascade in Plant Immunity

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B.Sc., Nankai University, 2013

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Botany)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

April 2016

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Abstract

Recognition of pathogens through pathogen-associated molecular patterns (PAMPs) or effectors in plants activates a variety of defense responses including MAPKs signaling pathways and defense related genes expression. ANPs (Arabidopsis Nucleus- and Phragmoplast- localized kinase 1 related protein kinases), including ANP1, ANP2, ANP3 are three MAP kinase kinase kinases that form a MAP kinase cascade with downstream MKK6 and MPK4 to regulate cytokinesis process. In this study, we showed that the anp2 anp3 double mutants exhibit constitutive expression of PR (Pathogenesis-Related) genes and enhanced resistance against oomycete pathogen H. a. Noco2, suggesting that ANP2 and ANP3 negatively regulate plant immunity. In addition, loss function of MKK6 causes high levels of PR gene expression, indicating that MKK6 is involved in negative regulation of defense responses. Since MPK4 was previously shown to function as a negative regulator of plant immunity, we tested whether MPK4 functions downstream of ANP2/ANP3 and MKK6 in plant immunity by introducing CA-MPK4 transgene, which expresses a constitutively active (CA) variant of MPK4, to anp2 anp3 and mkk6. Constitutive expression of PR genes and enhanced resistance to H.a. Noco2 in anp2 anp3 and mkk6 were partially suppressed by expressing CA MPK4, suggesting that the ANP2/ANP3, MKK6 and MPK4 function in a MAPK cascade to negatively regulate defense responses.

To find out components that function downstream of ANP2/ANP3-MKK6-MPK4 cascade in plant immunity, two mutants *summ2-8* (SUPPRESSOR OF MKK1 MKK2 2) and *pad4-1* (PHYTOALEXIN DEFICIENT 4) were crossed into *anp2 anp3* respectively. The constitutive defense responses in *anp2 anp3* were fully suppressed by *pad4-1*, but not affected by the *summ2-8* mutation, suggesting that PAD4 functions downstream of ANP2/ANP3 and that immune responses mediated by certain TIR-NB-LRR R proteins might be activated in the *anp2 anp3* mutant.

Preface

The work described in this thesis is the culmination of research from September 2013 through March 2016. Below is the manuscript (in preparation) that comprises this thesis and the contribution made by the candidate.

Chapter 2 – Arabidopsis MKK6 functions in two parallel MAP kinase cascades that negatively regulate plant immunity was modified from a prepared manuscript:

The work is done in collaboration with Fang Gao. The candidate performed the following experiments under the instruction of Yuelin Zhang: *PR* gene expression in *mkk6* and *CA-MPK4 mkk6*; characterization of *anp2 anp3*, *anp2 anp3 CA-MPK4*, *anp2 anp3 pad4-1* and *anp2 anp3 summ2-8*; *Pto DC3000 hrcC*⁻ infection assay. Fang Gao conducted the following experiments: characterization of *summ4-1D mkk1 mkk2*; positional cloning of *SUMM4*; *PR* gene expression in *summ4-1D*, *mekk1*, *mpk4*, *summ4-1D mekk1* and *summ4-1D mpk4*; interaction of MKK6 with MEKK1 and MPK4. Yuelin Zhang, Fang Gao and the candidate prepared the manuscript.

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List of Abbreviations

ANPs	Arabidopsis Nucleus- and Phragmoplast-
	localized kinase 1 related protein kinases
ASR3	Arabidopsis SH4-related 3
AvrB	an avirulence protein from Pseudomonas syringae
AvrRpm1	an avirulence protein from Pseudomonas syringae
AvrRpt2	an avirulence protein from Pseudomonas syringae
AvrPto	an avirulence protein from Pseudomonas syringae
AvrPtoB	an avirulence protein from Pseudomonas syringae
BAK1	BRI1-Associated Kinase 1
BiFC	Biomolecular Fluorescence Complementation
BIK1	Botrytis- Induced Kinase 1
BRI1	Brassinosteroid-Insensitive 1
CA	Constitutively active
CaMV 35S promoter	a very strong constitutive promoter found in
	Cauliflower mosaic virus
CC	Coiled-coil
CERK1	Chitin Elicitor Receptor Kinase 1
CFU	Colony-Forming Units
Co-IP	Co-Immunoprecipitation
Col-0	an Arabidopsis ecotype; it's referred as wild type in
	this thesis
DAMP	Danger- Associated Molecular Pattern
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EDS1	Enhanced Disease Susceptibility 1
EFR	EF-Tu Receptor

EF-Tu	bacterial translation elongation factor Tu
ERECTA	Receptor Protein Kinase
EMS	Ethyl Methanesulfonate; a chemical mutagen
ETI	Effector-triggered immunity
flg22	flagellin conserved domain of 22 amino acids
FLS2	Flagellin-Sensitive 2
<i>H.a.</i> Noco2	Hyaloperonospora arabidopsidis Noco2
HopAI 1	an avirulence protein from Psudomonas syringae
LysM	Lysine-motif protein
LRR	Leucine-rich repeat
МАРК	Mitogen-Activated Protein Kinase
MAP2K	MAP kinase kinase
МАРЗК	MAP kinase kinase kinase
MEKK	a MAP3K
МКК	MAP kinase kinase
МРК	MAP kinase
NACK1	NPK1-activating kinesin-like protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NAG	N-acetyl-D-glucosamine
NB	Nucleotide Binding
NDR1	Non-race-specific Disease Resistance 1
NPK1	Nucleus- and Phragmoplast- localized kinase 1
NQK1	a MAP2K in tobacco; orthologue of AtMKK6
NRK1	a MAPK in tobacco; downstream of NPK1 and
	NQK1
OGs	Oligogalacturonides
Os	Oryza sativa

PAD	Phytoalexin Deficient
PAMP	Pathogen-Associated Molecular Pattern
PCR	Polymerase Chain Reaction
<i>PR</i> gene	Pathogenesis-Related gene
PRR	Pattern Recognition Receptor
PBL27	PBS1-Like 27
PBS1	AvrPphB Susceptible 1
PTI	PAMP-triggered immunity
Pto	Pseudomonas syringae pv tomato
R protein	Resistance protein
RBOH	Respiratory Burst Oxidase Homolog
RIN4	RPM1-Interacting Protein 4
RLCK	Receptor-Like Cytoplasmic Kinase
RLK	Receptor-Like Kinase
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPM1	Resistance to Pseudomonas syringae pv
	Maculicola
RPS2	Resistant to Pseudomonas syringae 2
SA	Salicylic acid
SUMM	Suppressor of mkk1 mkk2
TIR	Toll Interleukin-1 Receptor
TTSS	Type Three Secretion System
Yoda	an Arabidopsis MAPK

Acknowledgements

This project was done with a lot of assistance from my colleagues and friends. First, I would like to thank my supervisor, Dr. Yuelin Zhang. He gave me this opportunity to work in his lab and he is so patient to guide and support me in my projects. The advices he provided are extremely valuable to me and facilitate to build up my scientific career.

I would like to thank my committee members, Dr. Xin Li and Dr. Jae-Hyeok Lee, for their encouragement and suggestions during my committee meetings.

I would like to thank previous lab member Dr. Pingtao Ding for his training and guiding at the start of my projects.

I would also like to appreciate all the members in Zhang lab and Li lab, who give me a lot of help not only in my work but also in my daily life. Yanan Liu, Tongjun Sun and Yuli Ding always gave me helpful suggestions for my project. Yukino Nitta helped me to revise my thesis. I collaborated with Fang Gao to finish the work in manuscript (Chapter 2), which will be used in her thesis.

I want to thank Botany Department for offering me TA positions. I gained a lot of teaching skills from this job.

Finally, I want to thank my family. They support my life in Vancouver and they always encourage me to pursue things that I want to do. I really appreciate for their patience and understanding.

Chapter 1 Introduction

1.1 Plant immunity

Living in an environment with diverse microbes, plants are challenged by pathogens all the time. Every year, there are a great number of crops affected by pathogen infections. This causes large impacts on human activity and economic development. Therefore, protection of crops against pathogens becomes an important topic widely studied by researchers.

Most pathogens have evolved to access the plant interior, either by penetrating the leaf or root surface directly or by entering through open areas on the plants, such as natural openings like stomata and wounds. Fungi can extend hyphae through plant cells, while nematodes and aphids can insert a stylet into plant cells directly. During infection, pathogens can secret effectors into plants to enhance their fitness (Jones and Dangl 2006).

However, plants have also evolved strategies to protect themselves. Unlike animals, plants do not have the adaptive immune system. Instead, they rely on their innate immunity to combat pathogen infections. There are two types of innate immune responses in plants: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered Immunity (ETI).

1.2 PAMP-triggered immunity

PAMP- triggered immunity acts as the first layer in the plant immune system. The pattern recognition receptors (PRRs), which localize on the plasma membrane, can perceive conserved microbial components collectively known as PAMPs. Some examples of well characterized PAMPs are flagellin, EF-Tu and chitin, while the corresponding pattern recognition receptors are FLS2, EFR and CERK1.

1.2.1 PRRs and PAMPs

1.2.1.1 FLS2 and flg22

FLAGELLIN SENSING 2 (FLS2) has been well studied as a receptor-like kinase (RLK), which contains an extracellular domain with 28 Leucine-Rich Repeats (LRRs), a transmembrane domain and an intracellular Ser/Thr protein kinase domain (Gomez-Gomez and Boller 2000). FLS2 can perceive the conserved 22-amino acid epitope flg22 on bacterial flagellin (Felix, Duran et al. 1999).

Upon flg22 perception, FLS2 can form a complex with another RLK, BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTOR KINASE1 (BAK1), which is also an important component involved in plant immunity (Chinchilla, Zipfel et al. 2007). A structural study revealed that the N terminus of flg22 binds to the surface of the FLS2 extracellular domain spanning 14 LRRs (LRR3-16 from N terminus of FLS2). The flg22-bound FLS2 ectodomain directly interacts with the BAK1 ectodomain with the C-terminus of flg22 stabilizing the dimerization of FLS2-BAK1, which acts as a molecular glue between two ectodomains (Sun, Li et al. 2013).

1.2.1.2 EFR and EF-Tu

The *fls2* mutant shows insensitivity to flg22 treatment, while when treated with crude bacterial extracts, defense responses were still detected. This indicates that there are additional PAMPs in bacteria, other than flg22. To identify these PAMPs, the crude extracts of *E.coli* Gl826 (FliC⁻), which lacks flagellin, was used to isolate and purify new elicitors. By analyzing the PAMP activity of each fraction of crude bacterial extracts, elongation factor-Tu (EF-Tu) was identified as a PAMP. The EF-Tu sequences are highly conserved amongst various bacterial species. The elicitor activity was shown in the 18 amino acids at the N terminus of EF-Tu. Thus, this synthetic peptide named elf18 was widely used as a PAMP (Kunze, Zipfel et al. 2004).

To identify the receptor of EF-Tu, a screen of T-DNA insertion lines of RLKs which were induced by either flg22 or EF-Tu was performed. EF-Tu Receptor (EFR) was identified as its T-DNA insertion line was insensitive to EF-Tu treatment. To further confirm that EFR is specific to EF-Tu, a complementation assay was done in the tobacco system. *Nicotiana benthamiana* lacks the ability to perceive EF-Tu, but showed defense response when *EFR* was transient transformed (Zipfel, Kunze et al. 2006).

1.2.1.3 CERK1 and chitin

Chitin, a polymer of N-acetyl-D-glucosamine (NAG), is a major component of fungal cell wall and acts as a PAMP. CERK1 (Miya, Albert et al. 2007) and LysM RLK1 (Wan, Zhang et al. 2008) were identified as the receptor of chitin in *Arabidopsis*.

CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) is a plasma membrane localized protein with three lysine motif (LysM) domains, which were found in a variety of enzymes involved in bacterial cell wall degradation and have a general ability to bind peptidoglycan (Visweswaran, Leenhouts et al. 2014). CERK1 contains an intracellular Ser/Thr kinase domain with autophosphorylation / myelin basic protein (MBP) kinase activity (Miya, Albert et al. 2007). A structural study revealed the interaction between chitin and CERK1. The dimerization of CERK1 ectodomain was induced by chitin, which acts as a bivalent ligand and this dimerization is critical for CERK1 activation (Liu, Liu et al. 2012).

1.2.2 Downstream defense responses

Upon perception of PAMPs by PRRs, several chemical and physical defense mechanisms are activated in plants to protect against pathogen infection, such as callose deposition, ROS production, Ca²⁺ influx and MAPK activation.

1.2.2.1 Callose deposition

Callose is a high-molecular weight (1,3)-β-glucan cell wall polymer. Callose

deposition, as a physical barrier between the plasma membrane and cell wall, can be induced in response to pathogen attack to prevent invading pathogens at the infection site (Nishimura, Stein et al. 2003). Therefore, callose deposition has been an effective response marker for studying signaling pathways involved in plant immunity.

1.2.2.2 ROS production

Reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), are produced as signaling messengers in stress response. ROS can be produced in the chloroplast, peroxisomes and mitochondria. In plants, ROS are proposed to act as antimicrobial and cross-linkers of the plant cell wall to prevent pathogen invasion (Lamb and Dixon 1997). ROS are produced by NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADPH) oxidases, which belong to the RESPIRATORY BURST OXIDASE HOMOLOG (RBOH) family. ROS production is considered one of the early responses induced by PAMPs in plant immunity. Accumulation of ROS can be observed after flg22 and elf18 treatment, while in *fls2* or *efr* mutants, no ROS accumulation can be detected after treatment with flg22 or elf18 (Zipfel, Kunze et al. 2006).

1.2.2.3 MAPK cascade activation

Mitogen-activated protein kinase (MAPK) cascades amplify signals from upstream stimuli through three types of phosphorylated kinases. The sequential phosphorylation starts from the MAP kinase kinase kinases (MAP3Ks), which can phosphorylate two Ser/Thr residues in the activation loop (Ser/Thr-X₃₋₅-Ser/Thr motif) of MAP kinase kinases (MAP2Ks). Then the MAP2Ks phosphorylate a Thr and Tyr residue in the Thr-X-Tyr activation loop of MAP kinases (MAPKs). The activated MAPKs then target downstream proteins to regulate biological processes (Meng and Zhang 2013). In Arabidopsis, there are approximately 60 MAP3Ks, 10 MAP2Ks and 20 MAPKs based on the genome sequence (Asai,

Tena et al. 2002).

An immunity-related MAPK cascade, which consists of MEKK1, MKK1 MKK2 and MPK4, was reported to negatively regulate defense responses (Gao, Liu et al. 2008). The roles of MEKK1, MKK1 MKK2 and MPK4 in plant immunity were revealed by their corresponding mutants. Both *mekk1* and *mpk4* single mutants show dwarfism and high H₂O₂ accumulation as well as extensive cell death and constitutive *PR* genes expression (Petersen, Brodersen et al. 2000, Ichimura, Casais et al. 2006, Gao, Liu et al. 2008). *MKK1* and *MKK2* are functionally redundant and the *mkk1mkk2* double mutants showed similar immune phenotypes as *mekk1* and *mpk4*, exhibiting high levels of H₂O₂ accumulation, extensive cell death and constitutive expression of *PR* genes (Gao, Liu et al. 2008, Qiu, Fiil et al. 2008). Additionally, the interactions between MEKK1, MKK1/MKK2 and MPK4 were examined using bimolecular fluorescence complementation (BiFC) assay. Interaction between MEKK1 and MKK1/MKK2 was found to interact in the plasma membrane, while MPK4 and MKK1/MKK2 was found to interact in the nucleus and plasma membrane(Gao, Liu et al. 2008).

To investigate the mechanism how MEKK1-MKK1 MKK2-MPK4 cascade regulates plant immunity, a suppressor screen of *mkk1 mkk2* was carried out. Suppressors of *mkk1 mkk2* (SUMMs) were identified through restored sizes of *mkk1mkk2*. *SUMM2* encodes a coiled-coil NB-LRR type R protein and the mutation in *SUMM2* can suppress cell death, accumulation of H₂O₂ and elevated *PR* genes expression in *mkk1 mkk2*. The constitutively active defense responses in *mekk1* and *mpk4* are also largely reduced by *summ2*. The pathogenic effector HopAI1 from *Pseudomonas syringae* was found to target MPK4 and block its kinase activity. Then it activates SUMM2-mediated defense responses.(Zhang, Wu et al. 2012)

Another *mkk1/mkk2* suppressor, SUMM1, was also identified and it encodes

MEKK2 which is a MAP3K. The constitutive activation of defense responses in *mekk1 mkk1/mkk2* and *mpk4* is largely blocked by the mutation in *MEKK2*. MPK4 and MEKK2 interact in the Yeast-two hybrid and co-immunoprecipitation (Co-IP) assays. Overexpressing MEKK2 also causes activation of cell death and defense responses. However, defense responses are not activated when MEKK2 was overexpressed in the *summ2* mutant background, indicating that constitutive defense responses in the MEKK2 overexpression lines are dependent on SUMM2 (Kong, Qu et al. 2012). Therefore, the MEKK1-MKK1/MKK2-MPK4 cascade negatively regulated defense responses by inhibiting MEKK2, which triggers SUMM2-mediated immune responses (Kong, Qu et al. 2012, Zhang, Wu et al. 2012).

Another MAPK cascade consisting of an unknown MAP3K, MKK4/MKK5 and MPK3/MPK6 was reported to act upstream of PHYTOALXIN DEFICIENT 2 (PAD2) and PAD3 to positively regulate biosynthesis of the phytoalexin camalexin (Ren, Liu et al. 2008). Reduced resistance against *Botrytis cinerea* and compromised camalexin accumulation were shown in *mpk3* and *mpk6* mutants, supporting the positive role of this cascade in camalexin biosynthesis in plants when challenged by pathogens (Ren, Liu et al. 2008).

1.2.3 Signaling components downstream of PAMP receptors

Receptor-like kinases (RLKs) are transmembrane proteins with N-terminal extracellular domains and C-terminal intracellular kinase domains. Plants have evolved a large repertoire of RLKs that form the largest family of plant membrane receptors with 610 members in Arabidopsis and 1,100 members in rice (Shiu, Karlowski et al. 2004). Some of the well-studied PAMP receptors are RLKs.

Plants have also evolved a large number of receptor-like cytoplasmic kinases (RLCKs), with 147 members in Arabidopsis and 379 members in rice (Shiu and Bleecker 2001, Vij, Giri et al. 2008). RLCKs are potentially anchored to the

plasma membrane through N-terminal putative myristoylation or palmitoylation motif. Lacking extracellular domain and transmembrane domain, RLCKs exclusively contain a Ser/Thr-special cytoplasmic kinase domain and more likely function to transduce signal rather than ligand perception (Lin, Ma et al. 2013).

One well studied RLCK is BOTRYTIS-INDUCED KINASE 1 (BIK1). *BIK1* was identified as an early induced gene in response to infection by *Botrytis cinerea*. *BIK1* was shown to play important roles in mediating PTI activated by multiple PAMPs. Based on *in vitro* assays, a general model of BIK1 in flagellin signaling was proposed (Lu, Wu et al. 2010, Zhang, Li et al. 2010). Upon flagellin binding to FLS2, flg22 induces FLS2 and BAK1 association and phosphorylation. The activated BAK1 phosphorylates BIK1, which in turn transphosphorylates the FLS2-BAK1 complex. The active BIK1 is likely disassociated from the FLS2-BAK1 complex to activate downstream intracellular signaling (Lu, Wu et al. 2010, Zhang, Li et al. 2010). Recent studies showed that activated BIK1 directly phosphorylated RBOHD, which leads to ROS production (Kadota, Sklenar et al. 2014, Li, Li et al. 2014). These evidences support that BIK1 is a critical regulator in the pathway from PAMP perception to downstream signal transduction.

PBS1-LIKE 27 (PBL27) was identified as an Arabidopsis orthologue of OsRLCK185 which was reported to interact with OsCERK1. Knockout mutant of *PBL27* resulted in the reduction of chitin-induced callose deposition and resistance against fungal infection. CERK1 was found to preferentially phosphorylate PBL27 rather than BIK1, while phosphorylation of PBL27 was not induced by flg22 treatment, suggesting that the signaling mediated by PBL27 is specific to CERK1 (Shinya, Yamaguchi et al. 2014).

1.3 Effector-triggered susceptibility

Successful pathogens deliver effectors to inhibit PTI responses using the Type Three Secretion System (TTSS) to promote bacterial growth. Some

effectors have been shown to target cell components that are involved in immune responses.

Two unrelated effectors secreted from *Pseudomonas syringae* AvrB and AvrRpm1 suppress PTI by promoting phosphorylation of RIN4, which acts as a negative regulator in PTI. Additionally, a third effector AvrRpt2, which is a cysteine protease, was reported to cleave RIN4 (Mackey, Holt et al. 2002, Axtell and Staskawicz 2003, Mackey, Belkhadir et al. 2003, Kim, Desveaux et al. 2005, Chung, da Cunha et al. 2011, Liu, Elmore et al. 2011). *Pseudomonas syringae* can secret other two effectors, AvrPto and AvrPtoB, to enhance their virulence by targeting BAK1 and disrupting flagellin-induced FLS2-BAK1 complex (Shan, He et al. 2008).

1.4 Effector-triggered immunity

Pathogen effectors can be perceived by resistance (R) proteins in plant. Most R genes encode Nucleotide Binding (NB)-Leucine Rich Repeat (LRR) proteins. After perception of effectors, R proteins usually trigger strong defense responses, which is effector-triggered immunity (ETI) (Jones and Dangl 2006).

Plant NB-LRR R proteins can be divided into two groups based on the sequence of N-terminal regions: Coiled-Coil (CC) NB-LRR and Toll Interleukin-1 Receptor (TIR) NB-LRR. The N-terminal domains may allow R proteins to interact with different targets in order to activate various signaling pathways. Effectors are recognized by R proteins either through directly binding to receptors or facilitation of an accessory protein (Jones and Dangl 2006). For example, phosphorylation of RIN4 by AvrB or AvrRpm1 can be perceived by the coiled-coil R protein RPM1 to activate RPM1-mediated immune responses (Chung, da Cunha et al. 2011, Liu, Elmore et al. 2011). The cleavage of RIN4 by AvrRpt2 can be sensed by another coiled-coil R protein RPS2, which activates RPS2-mediated defense responses (Axtell and Staskawicz 2003, Mackey, Belkhadir et al. 2003).

1.4.1 R protein signaling

Several genes required for R protein-mediated signaling pathway have been identified. Loss of function of NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) shows susceptibility to *Pseudomonas syringae* pv. *tomato* strain DC3000 carrying avirulence genes, *avrB, avrRpm1, avrRpt2* and *avrPph3*, which are recognized by coiled-coil NB-LRR proteins (Century, Holub et al. 1995), suggesting that NDR1 is required for the signaling pathway activated by CC-NB-LRR R protein. ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) were identified as positive regulators of the TIR-NB-LRR protein-mediated signaling pathway (Parker, Holub et al. 1996, Falk, Feys et al. 1999, Jirage, Tootle et al. 1999). Both *EDS1* and *PAD4* encode lipase-like proteins and they act as positive regulators in SA production (Jirage, Tootle et al. 1999, Feys, Moisan et al. 2001). EDS1 and PAD4 interact with each other and the interaction is required for TIR-NB-LRR R protein-mediated disease resistance (Feys, Moisan et al. 2001, Wagner, Stuttmann et al. 2013).

1.5 Roles of ANPs in plant development and immunity

1.5.1 ANPs regulate cytokinesis process

ARABIDOPSIS NUCLEUS- AND PHRAGMOPLAST-LOCALIZED KINASE 1 RELATED PROTEIN KINSES (ANPs) are a subfamily of MAP3Ks which includes three members, ANP1, ANP2 and ANP3. They were originally identified as orthologues of NUCLEUS- AND PHRAGMOPLAST-LOCALIZED KINASE 1 (NPK1) in tobacco (Nakashima, Hirano et al. 1998).

NPK1 localizes to the phragmoplast during cytokinesis and cytokinesis defective phenotypes such as multinucleate cells with incomplete cell plate and inhibition of phragmoplast expansion were observed when a kinase-negative version of NPK1 was expressed (Nishihama, Ishikawa et al. 2001). NPK1 regulates cytokinesis in tobacco through a MAPK cascade, which includes NPK1

(MAP3K), NQK1 (MAP2K) and NRK1 (MAPK). This cascade is activated by binding of kinesin-like proteins NPK1-activating kinesin-like protein 1 (NACK1) and NACK2 to NPK1 at the late mitotic phase of the cell cycle (Nishihama, Soyano et al. 2002, Takahashi, Soyano et al. 2004).

Arabidopsis *anp1*, *anp2* and *anp3* single knockout mutants have no obvious development phenotypes. The *anp2 anp3* double mutant has reduced plant size (Krysan, Jester et al. 2002). The hypocotyls and petals in *anp2 anp3* consist of a number of irregularly shaped cells with reduced elongation and radial swelling. No developmental defects were found in *anp1 anp2*, while only abnormal floral organs were showed in *anp1 anp3*. The *anp1 anp2 anp3* triple mutant cannot be obtained by crossing, indicating that these three genes are collectively essential in Arabidopsis (Krysan, Jester et al. 2002).

1.5.2 Roles of ANPs in immune responses

Several stress related genes, including pathogen response related genes and oxidative stress related genes, were found to be upregulated in the *anp2 anp3* double mutant based on microarray data (Krysan, Jester et al. 2002). To clarify the roles of *ANPs* in plant defense, the conditional silencing plants were generated by expressing *ANP3*- and *ANP1*- specific artificial microRNAs under the control of a β -estradiol inducible promoter in the *anp1 anp2* or *anp2 anp3* double mutant background (Savatin, Bisceglia et al. 2014). The ROS production was significantly reduced in the *anp2 anp3* double mutant and conditional triple mutants with the treatment of DANGER- ASSOCIATED MOLECULAR PATTERN (DAMP) oligogalacturonides (OGs) and PAMP elicitor elf18. In contrast, transgenic lines overexpressing *ANP1* or *ANP3* under the control of CaMV 35S promoter showed increased induction of ROS in response to OGs and ef118. In addition, a small reduction of OGs-induced MPK3/6 phosphorylation was also observed when the functions of ANPs were compromised. These data suggest

that ANPs play a positive role in elicitor-induced defense responses (Savatin, Bisceglia et al. 2014).

1.5.3 MKK6 and MPK4 function downstream of ANPs to regulate cytokinesis

A mutation in Arabidopsis *MKK6*, which is the orthologue of *NQK1* in tobacoo, causes dwarfism and large multinucleate cells with incomplete cell wall (Soyano, Nishihama et al. 2003), which is reminiscent of the mutant phenotype of *anps*. In addition, MKK6 was shown to localize at the equatorial plane of the phragmoplast (Takahashi, Soyano et al. 2010). MKK6 interacts with ANP3 in the nucleus in the BiFC assay (Zeng, Chen et al. 2011). In the presence of AtNACK1, ANPs can activate MKK6 in yeast (Takahashi, Soyano et al. 2010). These data suggest that MKK6 acts as the downstream MAP2K of ANPs in the regulation of cytokinesis.

Yeast two-hybrid analysis using the full set of AtMAP2Ks and AtMAPKs revealed that MKK6 can interact with MPK4, MPK11, MPK6 and MPK13 (Lee, Huh et al. 2008). These interactions were further confirmed by BiFC in Arabidopsis protoplasts (Zeng, Chen et al. 2011). In-gel kinase assays revealed MPK4 can be phosphorylated by MKK6 (Takahashi, Soyano et al. 2010). In a separate study, only MPK4 was strongly phosphorylated by constitutively active (CA) version of MKK6 (Zeng, Chen et al. 2011). These data suggest that MPK4 functions downstream of MKK6.

Similar to *mkk6* mutant, *mpk4* mutant plant shows a dwarf phenotype and exhibits abnormal cell grown patterns such as branching of root hairs and swelling of diffusely growing epidermal cells. These phenotypes are observed in the *anp2 anp3* double mutant (Beck, Komis et al. 2011). The cortical microtubule organization in the hypocotyl epidermal cells of *anp2 anp3* and *mpk4* was disoriented to form random net-like arrays instead of parallel arrays at the cell cortex (Beck, Komis et al. 2010). The microspore mother cells of *mkp4* failed to form a normal intersporal callose wall after male meiosis and the multinucleate

microspores undergo mitotic cytokinesis leading to enlarged pollen grains, which is reminiscent of the *mkk6* phenotype as well (Zeng, Chen et al. 2011). The similarities in *anp2 anp3, mkk6* and *mpk4* mutant phenotypes also suggest that MPK4 may form a MAPK cascade together with ANPs and MKK6 to regulate cytokinesis in Arabidopsis.

Taking all of these evidences together, we generate a hypothesis that ANP2/3-MKK6-MPK4 can form a cascade to regulate defense responses.

Chapter 2 Arabidopsis MKK6 functions in two parallel MAP kinase cascades that negatively regulate plant immunity

2.1 Summary

Arabidopsis MPK4 is a component of two independent MAP kinase cascades and functions in regulating development as well as plant defense. The MEKK1-MKK1/MKK2-MPK4 cascade inhibits activation of resistance protein-mediated defense responses mediated by SUMM2 and is also required for basal resistance, whereas the ANPs-MKK6-MPK4 cascade plays essential roles in cytokinesis. Here we report that MKK6 also functions together with MEKK1 and MPK4 to prevent activation of SUMM2-mediated immunity and the ANP2/ANP3-MKK6-MPK4 cascade negatively regulate a defense pathway that is independent of SUMM2. A gain-of-function mutant identified from a suppressor screen of mkk1mkk2 suppresses mkk1mkk2 but not mekk1 or mpk4 autoimmune phenotypes, suggesting that MKK6 shares similar functions as MKK1/MKK2 and works together with MEKK1 and MPK4 to inhibit activation of SUMM2-mediated defense responses. Interestingly, loss of MKK6 or ANP2 and ANP3 also causes constitutive activation of plant defense responses. The autoimmune phenotype in *mkk6* and *anp2* anp3 mutant plants can be largely suppressed by a constitutively active mutant of MPK4. Furthermore, constitutive defense responses in anp2 anp3 is dependent on the defense regulator PAD4, but not SUMM2, suggesting that the ANP2/ANP3-MKK6-MPK4 cascade negatively regulates а SUMM2-independent defense pathway.

2.2 Introduction

In Arabidopsis, there are 20 Mitogen-activated protein kinases (MAPKs), 10 MAPK kinases (MAPKKs) and about 60 MAPK kinase kinases (MAPKKKs) (MAPK-Group 2002). They work in combinations to form distinct MAP kinase cascades that play diverse roles in plant development and stress signaling (Rodriguez, Petersen et al. 2010, Meng and Zhang 2013). Several MAP kinase cascades including Yoda- MKK4/MKK5- MPK3/MPK6, MEKK1- MKK1/MKK2-MPK4 and ANPs (Arabidopsis NPK1-related Protein Kinases)- MKK6- MPK4 have been studied extensively.

Arabidopsis MKK4/MKK5 and MPK3/MPK6 function in regulating both development and defense against pathogens. They form a MAP kinase cascade with Yoda to mediate signal transduction from upstream RLKs such as ERECTA and BAK1 to the downstream transcription factors in stomata development (Bergmann and Sack 2007, Meng, Chen et al. 2015). In response to elicitor treatment, the MAP kinase cascade consisting of MKK4/MKK5, MPK3/MPK6 and an unknown MAPKKK are activated (Asai, Tena et al. 2002). This kinase cascade has been shown to play critical roles in the regulation of biosynthesis of ethylene and phytoalexin (Liu and Zhang 2004, Ren, Liu et al. 2008).

The MEKK1-MKK1/MKK2-MPK4 cascade is also activated following elicitor treatment (Gao, Liu et al. 2008, Qiu, Zhou et al. 2008). Components of this kinase cascade were originally identified as negative regulators of plant immunity based on the autoimmune phenotypes in the *mekk1*, *mkk1 mkk2* and *mpk4* mutants (Petersen, Brodersen et al. 2000, Ichimura, Casais et al. 2006, Nakagami, Soukupova et al. 2006, Suarez-Rodriguez, Adams-Phillips et al. 2007, Gao, Liu et al. 2008, Qiu, Zhou et al. 2008). Further studies on the suppressor mutants of *mkk1 mkk2* showed that autoimmunity in these mutants is caused by activation of the coiled-coil (CC)-nucleotide binding (NB)–Leu-rich repeat (LRR) protein

SUMM2 (Zhang, Wu et al. 2012). The autoimmune phenotypes in the *mekk1*, *mkk1 mkk2* and *mpk4* mutants are also dependent on MEKK2 (Kong, Qu et al. 2012, Su, Bush et al. 2013), but the mechanism is unclear. In the absence of SUMM2, *mekk1* and *mkk1 mkk2* mutant plants showed enhanced susceptibility to pathogens, suggesting that the MEKK1-MKK1/MKK2-MPK4 cascade functions in promoting basal resistance against pathogens (Zhang, Wu et al. 2012). Consistently, MPK4 is required for the induction of about 50% genes by flg22 (Frei dit Frey, Garcia et al. 2014). Interestingly, MPK4 also plays a role in the negative regulation of flg22-induced gene expression through phosphorylation of ASR3 (Li, Jiang et al. 2015).

From a functional yeast screen, mutations that render Arabidopsis thaliana MAPKs constitutively active have been identified (Berriri, Garcia et al. 2012). The specificity toward known activators and substrates appears to be unchanged in the constitutively active mutants of MAPKs (CA-MPKs). CA-MPK4 transgenic plants accumulate less salicylic acid following pathogen infection and exhibit enhanced susceptibility to pathogens (Berriri, Garcia et al. 2012). Interestingly, effector-triggered immunity specified by the Toll Interleukin-1 Receptor (TIR)–NB– LRR resistance proteins RPS4 and RPP4 were also found to be compromised in CA-MPK4 transgenic plants, suggesting that constitutive activation of MPK4 inhibits resistance mediated by RPS4 and RPP4.

ANP1, ANP2 and ANP3 are three MAPKKKs closely related to NPK1, which is involved in the regulation of cytokinesis in tobacco (Nishihama, Ishikawa et al. 2001). Single mutants of *anp1*, *anp2* and *anp3* have no clear phenotypes, whereas the *anp2 anp3* double mutant displays abnormal cytokinesis (Krysan, Jester et al. 2002). The *anp1 anp2 anp3* triple mutant cannot be obtained because of lethality. In Arabidopsis, MKK6 and MPK4 function downstream of ANPs to regulate cytokinesis (Beck, Komis et al. 2010, Kosetsu, Matsunaga et al. 2010, Takahashi, Soyano et al. 2010, Zeng, Chen et al. 2011). Loss of MKK6 or MPK4 leads to severe defects in cytokinesis. In this study, we report that MKK6 also functions together with MEKK1 and MPK4 to negatively regulate SUMM2-mediated immunity and the ANP2/ANP3-MKK6-MPK4 cascade plays a critical role in negative regulation of defense responses independent of SUMM2.

2.3 Results

2.3.1 Characterization of summ4-1D mkk1 mkk2

From a previously described suppressor screen of $mkk1 \ mkk2 \ (mkk1/2)$ (Zhang, Wu et al. 2012), we identified summ4-1D that suppresses the dwarf phenotype of mkk1/2 almost completely (Figure 1A). It was named summ4-1D because the mutation was later found to be dominant. To determine whether constitutive defense responses in mkk1/2 is suppressed by summ4-1D, we checked the expression levels of defense marker genes Pathogenesis-Related 1 (*PR1*) and *PR2* in $summ4-1D \ mkk1/2$. As shown in Figure 1B and 1C, constitutive expression of *PR1* and *PR2* in mkk1/2 is completely suppressed in the $summ4-1D \ mkk1/2$ triple mutant. We further tested whether $summ4-1D \ mkk1/2$ with the oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2. As shown in Figure 1D, growth of *H.a.* Noco2 on $summ4-1D \ mkk1/2$ was much higher than on mkk1/2 (Figure 1D). These data suggest that the constitutive defense responses in mkk1/2 are suppressed by the summ4-1D mutation.



Figure 1 Characterization of summ4-1D mkk1 mkk2.

(A) Morphological phenotypes of three-week-old wild type (WT), *mkk1 mkk2* and *summ4-1D mkk1 mkk2* plants.

(B-C) Expression levels of *PR1* (B) and *PR2* (C) in WT, *mkk1 mkk2* and *summ4-1D mkk1 mkk2* seedlings. Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations from three measurements.

(D) Growth of *H. a.* Noco2 on WT, *mkk1 mkk2* and *summ4-1D mkk1 mkk2* plants. Error bars represent standard deviations from three replicates. Statistical differences among the samples are labelled with different letters (P < 0.01, one-way ANOVA, n=3).

2.3.2 Positional cloning of SUMM4

In the F2 progeny of a cross between wild type and *summ4-1D mkk1/2*, plants homozygous for *mkk1/2* and heterozygous for *summ4-1D* were identified. These plants had wild type morphology, suggesting that the *summ4-1D* mutation is dominant. To map the *summ4-1D* mutation, *summ4-1D mkk1/2* was crossed with Landsberg *erecta* (L*er*). Plants that are *mkk1/2* homozygous in the F2 population were selected for linkage analysis. Crude mapping showed that the *summ4-1D* mutation is located between markers K19E20 and MMN10 on chromosome 5 (Figure 2A).

To identify the *summ4-1D* mutation, a genomic DNA library of *summ4-1D mkk1/2* was prepared and sequenced. Using single nucleotide polymorphisms identified from the sequence data and progeny of F2 plants homozygous for *mkk1/2* and heterozygous for *summ4-1D*, we further narrowed the *summ4-1D* mutation to a region between markers 22.8 and 23.5 on chromosome 5 (Figure 2A). Only one mutation, a C-to-T substitution located in the promoter region of *MKK6* (*At5g56580*), was identified in this region (Figure 2A).

To test whether this mutation causes suppression of the *mkk1/2* mutant phenotype, a genomic clone of *MKK6* carrying the candidate *summ4-1D* mutation was transformed into plants homozygous for *mkk1* and heterozygous for *mkk2*, as *mkk1/2* double mutant is seedling lethal. Transgenic plants homozygous for *mkk1* and *mkk2* were identified by PCR and they were found to display wild type-like morphology (Figure 2B), suggesting that the mutation in the promoter region of *MKK6* is indeed responsible for the suppression of the *mkk1 mkk2* mutant phenotypes in *summ4-1D mkk1/2*.





Figure 2 Positional cloning of SUMM4.

(A) Map position and the mutation in *summ4-1D*.

(B) Morphology of three-week-old *mkk1 mkk2* plants expressing *MKK6* driven by its native promoter containing the *summ4-1D* mutation. Three independent transgenic lines are shown.

(C) *MKK6* expression levels in WT, *mkk1 mkk2*, *summ2-8 mkk1 mkk2*, *summ4-1D mkk1mkk2* and *summ4-1D* seedlings. Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations from three measurements.

2.3.3 The summ4-1D mutation results in elevated expression of MKK6

Since the *summ4-1D* mutation is in the promoter region of *MKK6*, we tested whether the expression level of *MKK6* is affected. As shown in Figure 2C, the *summ4-1D mkk1/2* triple mutant has much higher expression of *MKK6* than wild type and *mkk1/2*. In contrast *summ2-8* does not affect the expression of *MKK6* in *mkk1 mkk2*. Similarly, the expression level of *MKK6* is also dramatically increased in the *summ4-1D* single mutant compared to wild type and *mkk1/2*, suggesting that the *summ4-1D* mutation causes increased *MKK6* expression.

2.3.4 *summ4-1D* does not suppress the autoimmune phenotypes of *mekk1* and *mpk4*

Since MEKK1 functions upstream of MKK1/MKK2, we generated the *summ4-1D mekk1-1* double mutant to test whether the *mekk1* mutant phenotype can be suppressed by *summ4-1D*. As shown in Figure 3A, *summ4-1D mekk1-1* has similar dwarf morphology as *mekk1-1*. The expression levels of *PR1* and *PR2* in the double mutant are also comparable to those in *mekk1-1* (Figure 3B and 3C), suggesting that *summ4-1D* cannot suppress the constitutive defense responses in *mekk1-1*.

We also generated the *summ4-1D mpk4-3* double mutant to test whether the *mpk4* mutant phenotype can be suppressed by *summ4-1D*. Morphologically the *summ4-1D mpk4-3* double mutant is indistinguishable from *mpk4-3* (Figure 3D). Analysis of the expression levels of *PR1* and *PR2* in *summ4-1D mpk4-3* showed that they are also similar to those in *mpk4-3* (Figure 3E and 3F), indicating that the autoimmunity phenotype in *mpk4-3* cannot be suppressed by the *summ4-1D* mutation.



Figure 3 *summ4-1D* does not suppress the autoimmune phenotypes of *mekk1* and *mpk4*.

(A) Morphology of three-week-old WT, *summ4-1D*, *mekk1-1* and *summ4-1D mekk1-1* plants.

(B-C) Expression levels of *PR1* (B) and *PR2* (C) in WT, *mekk1-1* and *summ4-1D mekk1-1* seedlings. Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations from three measurements.

(D) Morphology of three-week-old WT, *summ4-1D*, *mpk4-3* and *summ4-1D mpk4-3* plants.

(E-F) Expression levels of *PR1* (E) and *PR2* (F) in WT, *mekk1-1* and *summ4-1D mekk1-1* seedlings. Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations from three measurements.

2.3.5 MKK6 interacts with MEKK1 and MPK4

To test whether MKK6 interacts with MEKK1 and MPK4, split luciferase complementation assays were conducted using constructs expressing MKK6 fused to the C-terminal domain of luciferase (MKK6^{CLuc}) and MEKK1 and MPK4 fused to the N-terminal domain of luciferase (MEKK1^{NLuc} and MPK4^{NLuc}) under a 35S promoter. If MKK6 associates with MEKK1 or MPK4, a functional luciferase complex would be formed. Consistent with a previous report that MPK4 interacts with MKK6 in bifluorescence complementation assays, strong luciferase activity was observed when MKK6^{CLuc} and MPK4^{NLuc} were co-expressed in *Nicotiana* (*N*.) *benthamiana* (Figure 4). Luciferase activity was also observed when MKK6^{CLuc} and MEKK1^{NLuc} were co-expressed in *Nicotiana* (*N*.) *benthamiana* (Figure 4). Luciferase activity was also observed when MKK6^{CLuc} and MEKK1^{NLuc} were co-expressed in *Nicotiana* (*N*.) *benthamiana* (Figure 4). Luciferase activity was also observed when MKK6^{CLuc} and MEKK1^{NLuc} were co-expressed in *Nicotiana* (*N*.) *benthamiana* (Figure 4). Luciferase activity was also observed when MKK6^{CLuc} and MEKK1^{NLuc} were co-expressed in *Nicotiana* (*N*.) *benthamiana* (Figure 4). Luciferase activity was also observed when MKK6^{CLuc} and MEKK1^{NLuc} were co-expressed in *Nicotiana* (*Ni*) *benthamiana* (Figure 4). Luciferase activity was also observed when MKK6^{CLuc} and MEKK1^{NLuc} were co-expressed in *Nicotiana* (*Ni*) *benthamiana* (Figure 4). Luciferase activity was also observed when MKK6^{CLuc} and MEKK1^{NLuc} were co-expressed in *Nicotiana* (*Ni*) *benthamiana*, despite at lower levels (Figure 4). These data suggest that MKK6 interacts with both MEKK1 and MPK4 *in vivo*.

Figure 4 Interactions between MKK6 and MEKK1 or MPK4.

Quantitative analysis of luciferase activity, represent in Relative Light Units (RLU) of split luciferase complementation assay. Error bars represent standard deviations from eighet replicates. Statistical differences among the samples are labelled with different letters (P < 0.05, one-way ANOVA, n=8).

2.3.6 PR1 and PR2 are constitutively expressed in mkk6 mutant plants

To test whether defense responses are activated in *mkk6* mutant plants, we analyzed the expression of *PR1* and *PR2* in *mkk6-2*. As shown in Figure 5B and 5C, both *PR1* and *PR2* are constitutive expressed in *mkk6-2*. To determine whether constitutive activation of defense gene expression is caused by reduced MPK4 activity, we crossed *mkk6-2* with a transgenic line expressing the MPK4^{D198G/E202A} (CA-MPK4) mutant that is constitutively active and obtained the *mkk6-2 CA-MPK4* double mutant. Quantitative RT-PCR analysis showed that the constitutive expression of *PR1* and *PR2* in *mkk6-2* is largely blocked by the *CA-MPK4* transgene (Figure 5E and 5F), suggesting that MPK4 functions downstream of MKK6.

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Figure 5 Expression of *PR* genes in *mkk*6 and *CA-MPK4 mkk*6.

(A) Morphology of three-week-old WT and *mkk6* plants.

(B-C) PR1 and PR2 expression levels in WT and mkk6 seedlings.

(D) Morphology of three-week-old WT, *mkk6* and *mkk6* CA MPK4 plants.

(E-F) PR1 and PR2 expression levels in WT, mkk6 and CA-MPK4 mkk6 seedlings.

Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations from three measurements.

2.3.7 Defense responses are constitutively activated in the *anp2 anp3* double mutant

ANPs have previously been shown to interact with MKK6 and function upstream of MKK6 in regulating cytokinesis (Krysan, Jester et al. 2002, Beck, Komis et al. 2010, Kosetsu, Matsunaga et al. 2010, Takahashi, Soyano et al. 2010). Since microarray analysis showed that stress-related genes are up-regulated in the *anp2 anp3* double mutant (Krysan, Jester et al. 2002), we examined whether ANP2 and ANP3 are involved in negative regulation of plant immunity. Compared to wild type and the *anp2-2* and *anp3-3* single mutants, the *anp2-2 anp3-3* double mutant exhibits dwarf morphology (Figure 6A). Both *PR1* and *PR2* are constitutively expressed in the *anp2-2 anp3-3* double mutant, but not in the *anp2-2 anp3-3* exhibits enhanced pathogen resistance, it was challenged with *H. a.* Noco2. As shown in Figure 6D, *H. a.* Noco2 growth is greatly reduced in the *anp2-2 anp3-3* double mutant compared to those in the wild type and the single mutants, suggesting that ANP2 and ANP3 function redundantly in negative regulation of plant defense responses.

Figure 6 Characterization of the *anp2 anp3* double mutant.

(A) Morphology of three-week-old WT, *anp2-2, anp3-3* and *anp2-2 anp3-3* plants.
(B-C) *PR1* (B) and *PR2* (C) expression levels in WT, *anp2-2, anp3-3* and *anp2-2 anp3-3* seedlings.

(D) *H. a.* Noco2 growth on WT, *anp2-2, anp3-3* and *anp2-2 anp3-3* plants.
 Statistical differences among the samples are labelled with different letters (*P* <0.01, one-way ANOVA, n=3)

2.3.8 The autoimmune phenotype of *anp2 anp3* can be partially suppressed by the *CA-MPK4* mutant

To test whether the autoimmune phenotype in *anp2-2 anp3-3* is due to reduced activity of MPK4, the *anp2-2 anp3-3* double mutant was crossed with a transgenic line expressing the CA-MPK4 mutant to obtain the *anp2 anp3 CA-MPK4* triple mutant. As shown in Figure 7A, the dwarf morphology of *anp2 anp3* is partially suppressed by the CA-MPK4 mutant. Analysis of *PR* gene expression showed that the expression levels of both *PR1* and *PR2* are also lower in the *anp2 anp3 CA-MPK4* triple mutant (Figure 7B and 7C). In addition, growth of *H. a.* Noco2 is much higher in the triple mutant than in the *anp2 anp3* double mutant (Figure 7D). These data suggest that ANP2/ANP3 function upstream of MPK4.

Figure 7 *CA-MPK4* partially blocks the constitutive defense responses in *anp2-2 anp3-3*.

(A) Morphology of three-week-old WT, *anp2-2 anp3-3* and *CA-MPK4 anp2-2 anp3-3* plants.

(B-C) *PR1* (B) and *PR2* (C) expression levels in WT, *anp2-2 anp3-3* and *CA-MPK4 anp2-2 anp3-3* seedlings.

(D) *H. a.* Noco2 growth on WT, *anp2-2 anp3-3* and *CA-MPK4 anp2-2 anp3-3* plants. Statistical differences among the samples are labelled with different letters (P < 0.01, one-way ANOVA, n=3).

2.3.9 Constitutive defense responses in *anp2 anp3* are independent of *SUMM2*

As constitutive defense responses in *mpk4* are largely dependent on SUMM2, we tested whether the SUMM2-dependent defense pathway is activated in *anp2 anp3*. The *anp2-2 anp3-3 summ2-8* triple mutant was obtained by crossing *summ2-8* into *anp2-2 anp3-3*. As shown in Figure 8A, *summ2-8* has no effects on the morphology of *anp2-2 anp3-3*. In addition, *summ2-8* has no effects on the expression of *PR1* (Figure 8B) and *PR2* (Figure 8C) and resistance to *H. a.* Noco2 (Figure 8D), suggesting that the autoimmune phenotype of *anp2 anp3* is independent of SUMM2.

2.3.10 PAD4 is partially required for the autoimmune phenotype of *anp2 anp3*

Constitutive activation of MPK4 was previously shown to compromise effector-triggered immunity specified by the TIR-NB-LRR resistance proteins RPS4 and RPP4. To test whether resistance mediated by TIR-NB-LRR proteins is activated in *anp2 anp3*, we crossed a loss-of-function mutant of *PAD4*, which is required for resistance mediated by TIR-NB-LRR proteins (Glazebrook, Rogers et al. 1996, Feys, Moisan et al. 2001), into *anp2-2 anp3-3*. As shown in Figure 8E, the *pad4-1* mutation partially suppresses the dwarf morphology of *anp2-2 anp3-3*. Elevated expression levels of *PR1* and *PR2* in *anp2-2 anp3-3* are almost completely suppressed in the *anp2-2 anp3-3 pad4-1* triple mutant (Figure 8F and 8G). Furthermore, the enhanced resistance to *H. a.* Noco2 in *anp2 anp3* is also abolished in the *anp2 anp3 pad4-3* triple mutant (Figure 8H). These data suggest that the autoimmune phenotype of *anp2-2 anp3-3* is partially dependent on *PAD4*.

Figure 8 Constitutive defense responses in *anp2-2 anp3-3* are independent of *SUMM2* but dependent on *PAD4*.

(A) Morphology of three-week-old WT, *anp2-2 anp3-3* and *summ2-8 anp2-2 anp3-3* plants.

(B-C) *PR1* (B) and *PR2* (C) expression levels in WT, *anp2-2 anp3-3* and *summ2-8 anp2-2 anp3-3* seedlings.

(D) *H. a.* Noco2 growth on WT, *anp2-2 anp3-3* and *summ2-8 anp2-2 anp3-3* plants. Statistical differences among the samples are labelled with different letters (P < 0.01, one-way ANOVA, n=3).

(E) Morphology of three-week-old WT, *anp2-2 anp3-3* and *pad4-1 anp2-2 anp3-3* plants.

(F-G) *PR1* (F) and *PR2* (G) expression levels in WT, *anp2-2 anp3-3* and *pad4-1 anp2-2 anp3-3* seedlings.

(H) *H. a.* Noco2 growth on WT, *anp2-2 anp3-3* and *pad4-1 anp2-2 anp3-3* plants. Statistical differences are labelled with different letters (P < 0.01, one-way ANOVA, n=3)

2.3.11 *anp2-2 anp3-3* is more susceptible to *P. syringae* pv *tomato* DC3000 *hrcC*⁻

To test whether PTI is affected by loss of function of ANP2 and ANP3, *anp2-2*, *anp3-3* and *anp2-2 anp3-3* mutant plants were infiltrated with *Pto* DC3000 *hrcC*⁻, a bacterial strain deficient in delivery of type III effectors and often used as an indicator of PTI. As shown in Figure 9, growth of *Pto* DC3000 *hrcC*⁻ is comparable in *anp2-2*, *anp3-3* and wild type, but much higher in the *anp2-2 anp3-3* double mutant, suggesting that ANP2 and ANP3 function redundantly in the positive regulation of PTI.

Figure 9 Growth of *Pto* DC3000 *hrcC⁻* in wild type, *anp2-2*, *anp3-3*, and *anp2-2 anp3-3*.

Plants were infiltrated with *Pto* DC3000 $hrcC^{-}$ (OD₆₀₀ = 0.002). Bacterial growth was measured at day 0 and day 3 by taking leaf discs within the inoculated area. Statistical differences are labelled with different letters (*P*<0.01, one-way ANOVA, n=8).

2.4 Discussion

Despite that MEKK1 and MKK1/MKK2 function in the same MAP kinase pathway, the mutant phenotypes of *mekk1* and *mkk1 mkk2* are not identical (Rodriguez, Petersen et al. 2010). *mekk1* knockout mutant plants are much smaller than the *mkk1 mkk2* double knockout mutants, suggesting that one or more MKKs may have overlapping functions with MKK1/MKK2. We showed that MKK6 interacts with MEKK1 and MPK4 in split-luciferase assays and elevated expression of *MKK6* in the *summ4-1D* mutant suppresses the autoimmune phenotypes of *mkk1 mkk2*, but not those in *mekk1* and *mpk4*. These data suggest that MKK6 functions in parallel with MKK1/MKK2 to transduce signals from MEKK1 to MPK4 (Figure 10).

Arabidopsis ANPs and MKK6 have previously been shown to function together with MPK4 to regulate cytokinesis (Krysan, Jester et al. 2002, Beck, Komis et al. 2010, Kosetsu, Matsunaga et al. 2010, Takahashi, Soyano et al. 2010). Our data suggest that ANP2/ANP3 and MKK6 also play important roles in plant immunity. *anp2 anp3* and *mkk6* mutant plants constitutively express *PR* genes and exhibit enhanced pathogen resistance. These autoimmune phenotypes can be suppressed by a constitutively active MPK4 mutant protein, suggesting that ANP2/ANP3 and MKK6 function together with MPK4 in a MAP kinases cascade to negatively regulate plant defense (Figure 10).

Arabidopsis has 60 predicted MAPKKKs, but only 10 MKKs and 20 MAPKs (MAPK-Group 2002), suggesting that some of the MKKs and MAPKs may have multiple functions and can form distinct MAP kinase cascades with different MAPKKKs to regulate different biological processes. This is supported by the diverse roles of MKK4/MKK5 and MPK3/MPK6 in plant defense as well as in development (Meng and Zhang 2013). Our study revealed that MKK6 also has multiple functions. In addition to its roles in cytokinesis, MKK6 is also involved in

two MAPK kinase cascades that negatively regulate plant immunity.

Both the MEKK1-MKK1/MKK2-MPK4 and ANPs-MKK6-MPK4 cascades lead to activation of MPK4. Mutations in *summ2* suppress the autoimmune phenotypes of mekk1 and mkk1 mkk2, but not anp2 anp3, suggesting that these two MAP kinase cascades function independently in the negative regulation of plant immunity (Figure 10). This is consistent with that the mutant phenotypes of mekk1 and *mkk1 mkk2* are completely dependent on SUMM2, whereas the constitutive defense responses in *mpk4* can only be partially blocked by mutations in *summ2* (Zhang, Wu et al. 2012). It is unclear why two kinase cascades both leading to activation of MPK4 cannot compensate each other. Previously it was shown that MEKK1 interacts with MKK1 and MKK2 on the plasma membrane (Gao, Liu et al. 2008), whereas the ANPs-MKK6-MPK4 cascade functions in regulating cytokinesis in the nucleus (Beck, Komis et al. 2010, Kosetsu, Matsunaga et al. 2010, Takahashi, Soyano et al. 2010, Zeng, Chen et al. 2011). It is possible that the MEKK1-MKK1/MKK2-MPK4 and ANPs-MKK6-MPK4 cascades are active in different subcellular localizations to prevent constitutive activation of immune responses.

The mechanism of how the ANP2/ANP3-MKK6-MPK4 cascade negatively regulates plant immunity is unknown. Previously it was shown that expression of a constitutively active MPK4 leads to compromised pathogen resistance mediated by TIR-NB-LRR proteins (Berriri, Garcia et al. 2012). The autoimmune phenotype of *anp2 anp3* is dependent on PAD4, which is a critical positive regulator of TIR-NB-LRR protein mediated resistance (Glazebrook, Rogers et al. 1996, Feys, Moisan et al. 2001). It is likely that activation of MPK4 through the ANP2/ANP3-MKK6-MPK4 cascade is required for its functions in negative regulation of immunity mediated by one or more TIR-NB-LRR proteins.

Meanwhile, ANPs have been shown to function as positive regulators of

elicitor-triggered defense responses and protection against the necrotrophic fungus Botrytis cinerea (Savatin, Bisceglia et al. 2014). Increased growth of Pto DC3000 hrcC in the anp2-2 anp3-3 double mutant also supports a positive role of ANP2 and ANP3 in PTI. lt is likely that components of the ANP2/ANP3-MKK6-MPK4 cascade are targeted by certain pathogens and plants have evolved resistance proteins to sense disruption of this kinase cascade. Similar to protection of the MEKK1-MKK1/ MKK2-MPK4 cascade by the NB-LRR protein SUMM2 (Zhang, Wu et al. 2012), loss of function of ANP2/ANP3, MKK6 or MPK4 would result in activation of immunity mediated by the resistance proteins.

Figure 10 A working model for the roles of MKK6 in plant immunity.

MKK6 functions in parallel with MKK1 and MKK2 to form a MAPK cascade to prevent activation of SUMM2-mediated immunity. MKK6 also functions together with ANP2/ANP3 and MPK4 in a separate MAPK cascade that negatively regulates a PAD4-dependent defense pathway.

2.5 Methods

2.5.1 Plant materials

The summ4-1D mkk1/2 triple mutant was isolated from an EMS mutagenized M2 population of mkk1/2 (Zhang, Wu et al. 2012). mkk1-1 mkk2-1 (mkk1 mkk2 or mkk1/2), mpk4-3, mekk1-1, summ2-8, summ2-8 mkk1/2, mkk6-2, mkk6-3, pad4-1 and the CA-MPK4 transgenic line were described previously (Glazebrook, Rogers et al. 1996, Ichimura, Casais et al. 2006, Nakagami, Soukupova et al. 2006, Gao, Liu et al. 2008, Takahashi, Soyano et al. 2010, Berriri, Garcia et al. 2012, Zhang, Wu et al. 2012). The summ4-1D single mutant was isolated through backcrossing the triple mutant summ4-1D mkk1/2 to wild type Col-0 plants. The summ4-1D mekk1-1 double mutant was obtained by crossing summ4-1D mkk1/2 with *mekk1-1*. The summ4-1D mpk4-3 double mutant was obtained by crossing summ4-1D mkk1/2 with mpk4-3. The anp2-2 anp3-3 double mutant was obtained by crossing anp2-2 (Salk 144973) and anp3-3 (Salk 081990) obtained from the Arabidopsis Biological Resource Center. The anp2-2 anp3-3 CA-MPK4, anp2-2 anp3-3 summ2-8 and anp2-2 anp3-3 pad4-1 triple mutants were obtained by crossing anp2-2 anp3-3 with CA-MPK4CA, summ2-8 and pad4-1, respectively. Plants were grown at 23°C under 16 hr light/8 hr dark on soil or ½ Murashige and Skoog (MS) media.

2.5.2 Mutant characterization

To determine gene expression levels, RNA was extracted from two-week-old seedlings grown on ½ MS media using EZ-10 Spin Column Plant RNA Mini-Preps Kit (Bio Basic, Canada). Genomic DNA contamination was removed by treatment with RQ1 RNase-Free DNase (Promega). Reverse Transcription was carried out using M-MuLV reverse transcriptase (New England Biolabs). Real-time PCR was performed using SYBR Premix Ex Taq II (Takara). Each experiment was repeated with three independent RNA samples. Primers of *PR1*, *PR2* and *ACTIN1* used for

RT-PCR were previously described (Sun et al., 2015). Primers used for *MKK6* expression are listed in Supplemental Table 1.

H. a. Noco2 infection was performed on two-week-old seedlings. The seedlings were sprayed with spore suspensions at a concentration of 50 000 spores per ml water. The plants were covered with a clear dome and kept at 18°C under 12 h light/12 h dark cycles in a growth chamber. Samples were collected 7 days later and spores on the plants were resuspended in water and counted using a hemocytometer. Infection results were scored as previously described (Bi, Cheng et al. 2010).

P.syringae pv *tomato* DC3000 *hrcC*⁻ infection was performed on four-week-old plants grown in short-day conditions. Two leaves of each plant were infiltrated with *Pto* DC3000 *hrcC*⁻ (OD_{600} =0.002) and one leaf disc from each infiltrated leaf was collected. The two leaf discs from the same plants were mixed together and regarded as one sample. These samples were grinded and diluted, then plated on LB plates. Colony-forming units (CFU) were calculated by counting colonies.

2.5.3 Map-based cloning of SUMM4

For crude mapping of *summ4-1D*, the *summ4-1D mkk1/2* triple mutant was crossed with Landsberg *erecta* (L*er*). F2 plants homozygous for *mkk1/2* were selected for linkage analysis. *summ4-1D mkk1/2* was also crossed with wild type Col-0 plants to obtain the *summ4-1D* single mutant. Plants homozygous for *mkk1/2* and heterozygous for *summ4-1D* were also identified in the F2 generation and their progeny were used for fine mapping of *summ4-1D*. Markers for fine mapping were designed based on single nucleotide polymorphisms (SNPs) identified by sequencing the genome of *summ4-1D mkk1/2* using Illumina sequencing. All primer sequences are listed in Supplemental Table 1.

For testing whether the *summ4-1D* mutation is responsible for the suppression of the *mkk1/2* mutant phenotype, the *SUMM4* gene including the

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mutation in the promoter region was amplified from the genomic DNA of *summ4-1D mkk1/2* by PCR using primers MKK6-BamHI-F and MKK6-PstI-R. The DNA fragment was cloned into a modified pCambia1305 vector to express *MKK6* under the mutant version of its native promoter. The construct was transformed into plants homozygous for *mkk1* and heterozygous for *mkk2* by the floral dipping method (Clough and Bent 1998). Transgenic plants homozygous for *mkk1 mkk2* were identified by PCR in the T1 generation.

2.5.4 Split luciferase assay

For testing interactions between MKK6 and MEKK1 or MPK4, cDNA of MKK6 was amplified by PCR using primers MKK6-cLuc-F and MKK6-cLuc-R and cloned into pCamiba 1300 CLuc (Chen, Zou et al. 2008) to express MKK6^{CLuc} under a 35S promoter. cDNA fragments of MEKK1 and MPK4 were excised from pMEKK1-YCE and pMPK4-YCE (Gao, Liu et al. 2008) and cloned into pCamiba 1300 NLuc (Chen, Zou et al. 2008) to express MEKK1^{NLuc} and MPK4^{NLuc} under a 35S promoter. 30-day-old tobacco leaves were infiltrated with *Agrobacteria* (OD₆₀₀ = 0.2) carrying constructs expressing MKK6^{CLuc} and MEKK1^{NLuc} or MKK6^{CLuc} and MPK4^{NLuc}, along with the negative controls. Luciferase activity was measured using a plate reader. Plants were kept at 23°C under 16 hr light/8 hr dark condition for 2 days before assaying for luciferase activities.

Chapter 3 Future directions and conclusions

MAPK cascades play important roles in regulating a variety of biological processes in plants. The ANPs-MKK6-MPK4 cascade was originally proposed to regulate cytokinesis in Arabidopsis. My master thesis work suggested that this cascade also plays an important role in regulation of defense responses mediated by PAD4.

Defense responses in *anp2 anp3* and *mkk6* are only partially suppressed by introducing the constitutively active variant of MPK4. It's possible that the MPK4 activity in *CA MPK4* is lower than that activated by its upstream MAP2K, thus the defense responses in *anp2 anp3* and *mkk6* cannot be completely inhibited by *CA MPK4*. Alternatively, there could be other MAP kinases that function redundantly with MPK4 downstream of ANP2/ANP3 and MKK6.

Previous study showed that ANP3 can interact and phosphorylate MKK6 (Takahashi, Soyano et al. 2010, Zeng, Chen et al. 2011). To further confirm that MKK6 functions downstream of ANP2/ANP3 in plant immunity, a constitutively activate form of MKK6 will be transformed into *anp2 anp3* to test it can suppress defense responses in *anp2 anp3* plants.

PAD4 was found to function downstream of ANP2/ANP3, suggesting that one or more TIR-NB-LRR R proteins could function downstream of the ANP2/ANP3-MKK6-MPK4 cascade. To identify signaling components required for constitutive defense responses in *anp2 anp3*, a suppressor screen of *anp2 anp3* has been carried out. A number of putative suppressors with wild-type morphology were isolated. Further characterization of these suppressor mutants and isolation of the mutant gene by positional cloning will help us better understand the function of the ANPs-MKK6-MPK4 cascade in plant immunity.

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