2015

MAP Kinase Signaling in Plant Responses to Biotic Stress

Carlton James Bequette
University of South Carolina - Columbia

Follow this and additional works at: https://scholarcommons.sc.edu/etd

Part of the Biology Commons

Recommended Citation

This Open Access Dissertation is brought to you by Scholar Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact dillarda@mailbox.sc.edu.
MAP Kinase Signaling in Plant Responses to Biotic Stress

by

Carlton James Bequette

Bachelor of Science
University of South Carolina, 2007

Submitted in Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy in
Biological Sciences
College of Arts and Sciences
University of South Carolina
2015

Accepted by:
Johannes W. Stratmann, Major Professor
Anindya Chanda, Committee Member
Zhengqing Fu, Committee Member
Beth A. Krizek, Committee Member
Rekha C. Patel, Committee Member
Lacy Ford, Vice Provost and Dean of Graduate Studies
Acknowledgements

I am truly thankful for Dr. Johannes Stratmann for taking me on as a graduate student. He has been an incredibly supportive mentor and advisor throughout my graduate education. His guidance has not only enabled me to develop critical analytical skills, but also has encouraged me to become an independent researcher. I cannot thank Dr. Stratmann enough for everything he has taught me over the last six years. I would also like to thank my mentor, Dr. Sarah Hind, who was primarily responsible for my initial training and development. Her assistance during my first year was invaluable. Thank you to my fellow graduate student, Claire Hann, who I have enjoyed working alongside for the past few years. I would also like to thank Dr. Erika Balogh for her contributions to my research and the numerous undergraduate researchers who have contributed over the years.

A special thank you to the fellow graduate students from other laboratories who have provided fellowship and guidance along the way. I’d especially like to acknowledge Dr. Minsub Shim and Dr. Qian Wang, for graciously allowing me use of their equipment, and Dr. Michael Walla for his assistance with multiple projects.

I am truly and wholehearted thankful for my wonderful family that has supported me through my education. The most important person in my life and throughout this entire process has been my incredible wife, Mary Ellen. She has been a steady rock of support and encouragement. My kids are a blessing and provide me with endless enjoyment. They can always bring a smile to my face, and for that I am grateful.
Abstract

Plants must be able to perceive and properly respond to a multitude of environmental conditions and produce appropriate cellular responses for optimal growth and reproduction. Mitogen-activated protein kinase (MAPK) phosphorylation cascades are critical components of signal transduction networks that mediate cellular responses to a wide range of both biotic and abiotic extracellular stimuli. This thesis presents an investigation of three novel aspects of MAPK signaling, the role of MAPKs in the regulation of cell death, the organization of MAPKs in a multi-protein complex, and the negative regulation of MAPKs by MAPK phosphatases.

In tomato, three MAPKs play an essential role in many stress responses. When the genes for all three MAPKs are co-silenced, plants develop a spontaneous, runaway cell death phenotype. Necrotic lesions are associated with strong MAPK silencing, accumulation of catalase, and an elicitor-induced oxidative burst, that is stronger than in control plants. This indicates a novel role of MAPKs in the regulation of programmed cell death. In the second part of this research, a MAPK-containing multi-protein complex was characterized that is involved in the regulation of MAPK dephosphorylation. Typically, only inactive MAPKs associate with the complex. However, when cells were treated with a phosphatase inhibitor, active MAPKs associated with the complex. This suggests the involvement of MAPK phosphatases with the complex. The function of MAPK phosphatases in stress responses was further investigated in Arabidopsis MAPK phosphatase mutants. In these mutants MAPK signaling is altered in response to
wounding and a bacterial elicitor, and this correlates with altered defenses to insects and pathogenic bacteria.
# Table of Contents

Acknowledgements........................................................................................................... iii  
Abstract................................................................................................................................. iv 
List of Tables ........................................................................................................................... viii 
List of Figures ......................................................................................................................... ix 
Introduction................................................................................................................................ 1  
  Plant Responses to the Environment...................................................................................... 1  
  Plant Responses to Insect Herbivory....................................................................................... 2  
  Jasmonic Acid ........................................................................................................................ 4  
  Salicylic Acid and Plant Responses to Pathogens................................................................. 7  
  MAP Kinase Signaling Cascades............................................................................................ 8  

Chapter 1: The Role of Mitogen-Activated Protein Kinases in Plant Cell Death .............. 12  
  Abstract .................................................................................................................................... 13 
  Introduction ............................................................................................................................ 13 
  Methods ................................................................................................................................. 19 
  Results ................................................................................................................................. 27 
  Discussion .............................................................................................................................. 39 

Chapter 2: Identification of a MAP Kinase-containing Multi-protein Complex.............. 47  
  Abstract .................................................................................................................................... 48 
  Introduction ............................................................................................................................ 48
Methods .................................................................................................................53
Results ...................................................................................................................58
Discussion ...............................................................................................................68

Chapter 3: Characterization of Arabidopsis MAPK Phosphatases During Defense Responses ......................................................................................73
Abstract ...............................................................................................................74
Introduction .........................................................................................................75
Methods ............................................................................................................78
Results ...............................................................................................................80
Discussion .........................................................................................................86

Summary of Research Goals and Results ...............................................................90
References ...........................................................................................................93
List of Tables

Table 1.1: Sequences of primers used for pTRV2 vector construction ........................................26

Table 1.2: Sequences of primers used for RT-qPCR gene expression analysis .....................26

Table 3.1: Arabidopsis thaliana T-DNA insertion MAPK phosphatase null mutants and
            genotyping primers ........................................................................................................80
List of Figures

Figure 1.1: Spontaneous lesions in MPK1/2/3-VIGS plants ........................................28
Figure 1.2: Characteristics of cell death in lesion areas of MPK1/2/3-silenced plants......30
Figure 1.3: MPK1/2 protein levels and expression levels of MPK1, MPK2, and MPK3 in TS1-VIGS and TS2-VIGS plants.................................................................32
Figure 1.4: Increased ROS accumulation in MPK1/2/3-silenced plants after induction of an oxidative burst by Flg22.................................................................37
Figure 1.5: Decreased JA accumulation and increased SA accumulation in MPK1/2/3-silenced plants....................................................................................38
Figure 2.1: Identification of a MAP kinase-containing multi-protein complex in Arabidopsis, tobacco, and tomato.................................................................60
Figure 2.2: Active MPK1/2 does not associate with multi-protein complex in tomato ...62
Figure 2.3: Active MPK1/2 associates with a multi-protein complex after cantharidin treatment .................................................................................................64
Figure 2.4: Flow diagram for the identification of MAPK complex proteins .............66
Figure 2.5: Mass spectroscopic analysis of a MAPK complex candidate protein ..........67
Figure 3.1: Loss of MAPK phosphatases lead to prolonged MPK6 and MPK3 activation in response to wounding .................................................................82
Figure 3.2: JA accumulation at 1 h after wounding is not altered in MAPK phosphatase mutants....................................................................................83
Figure 3.3: Increased susceptibility in MAPK phosphatase mutants to fungal gnat larvae....................................................................................83
Figure 3.4: Loss of MAPK phosphatases lead to prolonged MPK6 activation in response to flg22 ....................................................................................85
Figure 3.5: SA Accumulation is not altered in MAPK phosphatase mutants ..........85
Introduction

Plant Responses to the Environment

Plants are sessile organisms that must be able to sense and adapt to the environment. There is a plethora of biotic and abiotic stresses plants may be exposed to during their lifetime, and they must be able to respond or adapt to stress in order to survive. Plants lack an acquired immune system containing antibodies and specific cells to defend themselves, however, they have developed a sophisticated innate immune system to rapidly perceive and respond to environmental stresses. In general, response to a changing environment involves recognition and subsequent transmission of the input signal via signal transduction cascades to induce the activation of specific output defense responses.

The main focus of this thesis will be on plant responses to biotic forms of stress, such as pathogenic microbes and herbivorous insects. Plants sense biotic stress through the recognition of stress-related non-host components, such as microbe/pathogen-associated molecular patterns (MAMPs or PAMPs), as well as herbivore-associated molecular patterns (HAMPs). MAMPs and HAMPs are elicitors produced by microbes or herbivores that activate host defense responses. The MAMP elicitor flg22 is a conserved 22 amino acid peptide from the bacterial flagellin protein, which triggers plant defenses upon perception (Gomez-Gomez and Boller, 2002). Plants are able to sense and activate defenses in response to HAMPs (Felton and Tumlinson, 2008), such as the fatty acid-amino acid conjugate volicitin (Alborn et al., 1997) and the peptide elicitor inceptin are
found in oral secretions of caterpillars (Schmelz et al., 2006). Plants can also recognize self-derived danger-associated molecular patterns (DAMPs), such as oligogalacturonic acid (OGA) fragments, which are derived from the plant cell wall during pathogen attack due to the breakdown of cell wall pectin by microbial polygalacturonase. OGA fragments are only present in cells being attacked by microbes and can induce plant defense responses such as an oxidative burst (Hahn et al., 1981; Galletti et al., 2008). In addition, plants can perceive other consequences of mechanical wounding or damage, such as electrical or hydraulic signals (Malone and Stankovic, 1991; Malone, 1992). Sensors or receptor proteins generally perceive these stress signals. For example, the MAMP flg22 is perceived by a membrane spanning receptor protein, FLS2, which interacts with flg22 via its extracellular domain, transmits the signal into the cell via its transmembrane domain, and initiates an intracellular signaling cascade via its intracellular kinase domain. This results in activation or mobilization of signaling events such as rapid membrane potential changes, calcium influx into the cell and activation of calcium-dependent protein kinases and/or mitogen-activated protein kinase (MAPK) cascades (Altenbach and Robatzek, 2007; Jeworutzki et al., 2010; Dangl and Jones, 2001). Downstream defense responses include the activation of defense genes. Signaling components are highly connected forming a signaling network that integrates input signals such that specific output responses can be induced.

Plant Responses to Insect Herbivory

One of the major environmental stresses to plants is damage and wounding due to feeding by phytophagous insects. Plants are able to sense and respond to the presence of insects through their movement (Bown et al., 2002; Peiffer et al., 2009), oviposition (Kim
et al., 2012), mechanical damage caused by feeding (León et al., 2000), and HAMPs (Alborn et al., 1997; Halitschke et al., 2001). Plants use both direct and indirect defense strategies to reduce the damage inflicted by feeding insects.

Direct defenses consist of physical barriers, such as trichomes, cuticles, and leaf toughness, and chemical defenses that reduce damage from feeding by exerting repellent, toxic, or antinutritive effects on herbivores (Bennett and Wallsgrove, 1994). Additionally, wounding by insects can stimulate the production of anti-digestive proteins, such as proteinase inhibitors, that function in the gut of the insect to reduce availability of essential amino acids (Koiwa et al., 1997; Tamayo et al., 2000). To defend themselves, plants have developed both mechanical and chemical defenses. Mechanical defenses are usually constitutively present physical structures such as thorns, trichomes and waxy cuticles. Chemical defenses can be both constitutive and/or inducible, such as the synthesis of toxic secondary metabolites and defense-related proteins. (Howe and Jander, 2008; Dodds and Rathjen, 2010). In addition, in response to pathogens, plant cells can undergo programmed cell death, which harms pathogen invaders while sacrificing healthy cells area the infection (Lam et al., 2001).

Indirect defenses involve the enemies of herbivores. The plant actively recruits those enemies via herbivore-induced emission of volatile compounds. For example, plants can release a specific volatile blend in response to feeding by a caterpillar, which attracts a parasitoid wasp, that ovideposits into the caterpillar. The developing wasp larvae reduce the fitness of the caterpillar resulting in reduced feeding and sometimes death of the caterpillar and ultimately, reduced damage to the plant (Mattiacci et al., 1995). The plant hormone jasmonic acid and its active derivatives are important wound
signaling components that are responsible for mediating the direct and indirect defense responses to insect herbivory.

**Jasmonic Acid**

Plants have a complex system of hormones that are involved in the regulation of plant growth, development, reproduction, and defense. Phytohormones are small compounds that act as signaling molecules and exist at low basal concentrations. Some of the classical plant hormones are auxin, abscisic acid, cytokinin, gibberellin and ethylene, and more recently additional plant hormones have been described such as brassinosteroids, jasmonate, salicylic acid, nitric oxide, and strigolactones. Another class of hormones that plants utilize to coordinate and direct cellular functions are secreted peptide hormones, such as the tomato wound signal systemin (Pearce et al., 1991). Defense-related peptide hormones are usually endogenous short peptides (18 to 23 amino acid residues) that are processed from larger polypeptide precursors in response to stress and induce downstream defense mechanisms (Bari and Jones, 2009; Yamaguchi and Huffaker, 2011).

Jasmonic acid (JA) and its bioactive derivatives, collectively referred to as jasmonates, are required for defense against herbivorous insects and necrotrophic pathogens (Howe et al., 1996). Jasmonates have been shown to act synergistically and antagonistically with other phytohormones such as ethylene (Creelman and Rao, 2002; Wasternack and House, 2002) and salicylic acid (Peña-Cortés et al., 1993; Sano et al., 1996, Van der Does et al., 2013). Both direct and indirect defenses are orchestrated by JA (Farmer and Ryan, 1990; Koch et al., 1999, Kessler et al., 2004; Wu and Baldwin, 2008). Additionally in tomato, JA functions as a long distance signal to stimulate JA-responsive
gene activation systemically in response to wounding (Li et al., 2002). MAP kinase signaling plays an important role in JA-mediated responses since silencing MPK1/2 or MPK3 in tomato or the tobacco homologs, WIPK and SIPK, decreases resistance to insect herbivores and reduces wound-induced synthesis of JA (Kandoth et al., 2007; Wu et al., 2007). In tobacco plants that have constitutive activation of WIPK, there is a three- to four-fold increase of jasmonate over wild type plants (Seo et al., 1999), which indicates that MAPK cascades function upstream of JA signaling and are required for proper JA-mediated responses.

Jasmonates are synthesized via the octadecanoid pathway, which initiates with the release of α-linolenic acid from plastid membrane glycerolipids by lipases (Hyun et al., 2008). The biosynthesis of JA initiates in the chloroplast where polyunsaturated fatty acids are converted to (9S,13S)-12-oxo-phytodienoic acid (OPDA). The remaining steps of the JA biosynthetic pathway occur in the peroxisome where OPDA is first reduced by OPDA reductase and then converted to JA by three rounds of β-oxidation (Vick and Zimmerman, 1983, Yan et al., 2013). It is not known how OPDA is released from the chloroplast and subsequently taken up by the peroxisome, however, there is evidence to suggest that the ABC transporter COMATOSE may be involved in this process (Theodoulou et al., 2005). JA can be further transformed by enzymatic conversion into numerous bioactive JA derivatives. Two of the major JA derivatives involved in defense responses are formed either through methylation to produce the volatile methyl ester, methyl-JA (Farmer and Ryan, 1990), or the conjugation of JA to isoleucine to form JA-Ile (Krumm et al., 1995; Kang et al., 2006; Howe and Jander, 2008). There are also alternative pathways to JA biosynthesis. For example, the linolenic acid analog,
hexadecatrienoic acid, is converted through a set of enzymatic steps to JA in a similar manner as linolenic acid (Yan et al., 2013).

During unstressed conditions where JA concentrations are low, transcription factors involved in promoting JA-induced gene expression, such as MYC2, are repressed by proteins of the JASMONATE ZIM domain (JAZ) family (Chini et al., 2007). Damage to the plant induces accumulation of JA-Ile, which stimulates the binding of JAZ proteins to the F-box protein CORONATINE INSENSITIVE1 (COI1). COI1 is part of the E3 ubiquitin ligase SCF^{COI1} which ubiquitinates JAZ proteins to target them for degradation by the 26S proteasome. Degradation of JAZ proteins alleviates the repression of transcription factors that promote JA-responsive gene expression (Thines et al., 2007). Null mutations in COI1 (coi1) in Arabidopsis plants have altered JA responses due to their inability to sense JA (Feys et al., 1994), and silencing COI1 in tomato and Nicotiana attenuata renders plants insensitive to JA and prevents the upregulation of JA-responsive defenses. This decreases resistance to herbivores and pathogens (Li et al., 2004; Paschold et al., 2008). The Ile-JA conjugate, and JA conjugates with structurally related amino acids, is responsible for stimulating the JAZ-COI1 interaction, but not methyl-JA or the JA precursor, OPDA (Thines et al., 2007; Katsir et al., 2008).

In addition to regulating defense responses, JA can be further catabolized by JA-carboxymethyl transferase (JMT) to form the volatile compound methyl-JA. This compound is produced following stress and is released by the plant where it may serve as a systemic volatile signal to the entire plant resulting in stimulation of JA-mediated responses. Once JA is methylated and volatilized, it travels to systemic parts of the plant where it can be converted back to JA via de-esterification by jasmonate methyl esterase.
and induce JA-dependent responses (Wu et al., 2008). Methyl JA serves to induce JA responses locally and systemically, but, as a volatile, it can also function in inter-plant communication and prime defense responses in neighboring plants for a possible attack (Arimura et al., 2000; Cheong and Choi, 2003).

**Salicylic Acid and Plant Responses to Pathogens**

Plants can recognize relatively large taxonomic groups of potential pathogens using cell-surface receptors that recognize highly conserved molecular structures, known as pathogen-associated molecular patterns (PAMPs). Recognition of a PAMP by a pattern-recognition receptor triggers an immune response, known as PAMP-triggered immunity (PTI), which includes callose deposition between the cell wall and the plasma membrane (Bestwick et al., 1995), activation of defense genes, and stomatal closure (Lee et al., 1999). Signaling events that mediate this response include a rapid oxidative burst in the apoplastic space (Meszaros et al., 2006; Nuhse et al., 2007; Zhang et al., 2007), ion fluxes across membranes, activation of MAP kinase and calcium-dependent protein kinase signaling cascades and activation of transcription factors involved in promoting defense genes (Jones and Dangl, 2006; Schwessinger and Zipfel, 2008; Mao et al., 2011), as well as induction of the plant defense hormone salicylic acid (Mishina and Zeier, 2007).

In the evolutionary arms race between plants and pathogens, pathogens have developed a strategy to suppress PTI by injecting effector proteins into the plant cell that block different aspects of PTI signaling (Jones and Dangl, 2006). For example, an effector from *Pseudomonas syringae*, AvrPtoB, can target the flagellin receptor FLS2 for degradation (Gohre et al., 2008). In turn, plants have evolved resistance (R) proteins that
can specifically recognize effector proteins in a gene-for-gene manner. Direct or indirect recognition of an effector protein by an R protein leads to activation of effector-triggered immunity (ETI), which leads to rapid programmed cell death at the site of infection. This defense, referred to as the hypersensitive response (HR), functions to kill the cells surrounding the infection to prevent the spread of the pathogen to neighboring cells (Levine et al., 1994; Caplan et al., 2008). The HR is induced in response to both biotrophic and necrotrophic pathogens, but is only functional in resistance against biotrophs (Mayer et al., 2001; van Kan, 2006). Recognition of a pathogen also induces the production of the hormone salicylic acid (SA) and its derivative methyl-SA, which leads to the systemic expression of pathogenesis-related (PR) genes in systemic uninfected tissues. This functions to protect the plant from subsequent infection and provides a broad-spectrum resistance to pathogens (Fu and Dong, 2013). This is known as systemic acquired resistance. Mutants defective in SA synthesis (ics1) or plants overexpressing the SA-degrading enzyme salicylate hydroxylase (Gaffney et al., 1993; Wildermuth et al., 2001) are defective in SAR. SA is required for proper defense responses to biotrophic and hemi-biotrophic pathogens (Glazebrook, 2005). It is not well known how recognition of a pathogen leads to SA production or the mobile signal for SAR.

**MAP Kinase Signaling Cascades**

Mitogen-activated protein kinase pathways are one of the main protein phosphorylation cascades in all eukaryotes. MAP kinase signaling pathways are responsible for translating environmental cues into specific cellular responses. MAPK pathways play a central role in the regulation of growth, development, cell death,
differentiation, proliferation, and stress responses (Nakagami et al., 2005; Mishra et al., 2006). MAPK pathways consist of three functionally related proteins, where the MAPK is phosphorylated and activated by a MAPK kinase (MAPKK), which itself is phosphorylated and activated by a MAPKK kinase (MAPKKK) (Rodriguez et al., 2010; Hamel et al., 2006). MAP kinase signaling modules can be linked in various arrangements with upstream receptors and downstream targets to transduce and amplify signals into specific responses (Cvetkovska et al., 2005). They are activated in response to various environmental abiotic stresses such as extreme temperatures, drought, salinity, and ultraviolet radiation, and biotic stresses such as wounding, herbivory, and pathogenic bacteria (Hirt, 2002; Jonak et al., 2002; Zhang and Klessig, 2001; Holley et. al., 2003; Nakagami et al., 2005; Kandoth et al., 2007). MAP kinases are also involved in response to endogenous stimuli and plant hormones such as ethylene and auxin (Xing et al., 2002; Zhang and Klessig, 2001; Rodriguez et al., 2010), and regulation of SA and JA biosynthesis and signaling (Kandoth et al., 2007; Seo et al., 2007).

The Arabidopsis genome contains 20 MAPKs, 10 MAPKK and ~80 putative MAPKKKs (MAPK Group, 2002), which implies convergence and divergence at the level of the MAPKK, where multiple MAPKKKs may activate the same MAPKK, which can then activate multiple MAPKs (Andreasson and Ellis, 2010). Based on sequence alignments of plant MAPKs there is a high degree of similarity in the kinase domains, whereas, the short N- and C-termini tend to be more variable. MAP kinases can be categorized into two groups based on the specific amino acid residues of the phosphorylation motif in the activation domain, which can be either Thr-Glu-Tyr (T-E-Y) or Thr-Asp-Tyr (T-D-Y). Some TEY MAPKs have been well characterized (e.g.
Arabidopsis MPK6, MPK4, and MPK3), however, little is know about the TDY MAPKs. During signal transduction, MAPKs must be dual phosphorylated on both the threonine and tyrosine in order to become active. This phosphorylation is reversible by the removal of one or both phosphate groups from the T-X-Y phosphorylation motif in the activation loop by MAPK phosphatases, which renders the kinase inactive (Camps et al., 2000). The role of MAPK phosphatases on the regulation of MAPK signaling will be further discussed in Chapter 3.

Two well-characterized MAPKs in plants are Arabidopsis are MPK6 (AtMPK6) and MPK3 (AtMPK3) and their tomato homologs SlMPK1 and SlMPK2 (homologs of AtMPK6), and Sl-MPK3 (AtMPK3 homolog). MPK6 and MPK3 are involved in the response to many biotic and abiotic stresses, especially in response to herbivory or mechanical wounding and response to pathogens (Andreasson and Ellis, 2010). Additionally, Arabidopsis MPK4 has also been shown to be an important negative regulator of SAR (Mou et al., 2003). AtMPK6 and AtMPK3 are associated with regulation of plant development and growth, e.g. stomatal patterning (Wang et al., 2007), floral organ abscission (Cho et al., 2008), and ovule development (Wang et al., 2008). Although AtMPK6 and AtMPK3 have different expression patterns in response to stresses, there is a degree of redundancy since Arabidopsis MPK3/MPK6 double mutants are embryo lethal (Wang et al., 2007 and 2008). Single mutants of either MPK6 or MPK3 are phenotypically similar to wild type plants (Bush and Krysan, 2007; Wang et al., 2007 and 2008).

Many components of MAPK signaling cascades are highly conserved in all eukaryotes, whereas, MAPK substrates are not conserved between mammalian, fungal
and plant systems. MAPKs are able to phosphorylate many substrates, such as transcriptional factors (Mao et al., 2011; Cheong and Choi, 2003), the ethylene biosynthetic enzyme ACC synthase 6 (Liu and Zhang, 2004), and cytoskeleton-associated proteins (Nakagami et al., 2005). A proteomics analysis using active MAPKs to probe high-density protein microarrays found many known and novel substrates able to be phosphorylated by MAPKs. Based on these data a signaling network consisting of nine MAPKKs, 10 MPKs, and 570 MAPK substrates was deduced (Popescu et al., 2009). Arabidopsis MPK3, MPK4, and MPK6 are promiscuous serine/threonine kinases that are associated with both defense responses and physiological processes. The same MAPK can be activated by multiple input signals, indicating tight regulation of MAPK signaling is necessary for proper signaling specificity. There are several different mechanisms that have been shown to be involved in determining the specificity of MAPK signaling: scaffolding proteins (so far only known in mammals and fungi), spatial and temporal coordinated expression of the MAPK and its substrate, specific MAPK docking domains, and/or residues surrounding phosphorylation sites within the MAPKs (Sorensson et al., 2012), and differences in the intensity and duration of MAPK activation (Bartels et al., 2010). Although there is evidence of various strategies of organization and coordination of MAPKs in animals and fungi, there is not much known about the organization of MAPK signaling modules in plants. This will be further discussed in Chapter 2.
Chapter 1: The Role of Mitogen-Activated Protein Kinases in Plant Cell Death
Abstract

Plants are generally exposed to a range of environmental stresses and stimuli and they must be able to appropriately perceive and coordinate proper responses for an optimal balance between growth, development, and defense. Mitogen-activated protein kinases (MAPKs) are part of phosphorylation cascades that regulate a number of cellular processes. Co-silencing of tomato MAPKs MPK1, MPK2, and MPK3, which are implicated in a number of defense responses, results in spontaneous, runaway cell death. Small lesions develop initially on any green, aerial portions of the plant and progress into massive necrotic lesions. Lesions were associated with the strongest reduction of MPK1/2 protein levels and MPK1, MPK2, and MPK3 expression levels, increased catalase accumulation, catalase activity, and CAT1 expression. The oxidative burst induced by the MAMP flg22 is significantly increased in amplitude and duration in MPK1/2/3-silenced plants compared to control plants. The wound-induced accumulation of the phytohormone jasmonic acid is reduced, and accumulation of salicylic acid is increased in wounded and unwounded plants. Increased SA accumulation in lesion areas is correlated to increased PR1a expression. Together, these results indicate that MPK1, MPK2, and MPK3 are involved in the negative regulation of HR-like cell death.

Introduction

MAP kinase signaling cascades

Protein phosphorylation is conserved in all eukaryotes and is one of the most common post-translational modifications involved in cellular functions. One family of protein kinases involved in many cellular responses is mitogen-activated protein kinases (MAPKs). MAPKs play a critical role in acting downstream of receptors to transmit
extracellular stimuli into cellular responses. MAPK signaling cascades consist minimally of a MAPK that is phosphorylated and activated by a MAPKK, which is phosphorylated and activated by a MAPKKK. MAPKs have been thoroughly characterized in plants and are involved in a diverse array of cellular functions, such as defense responses to biotic and abiotic stress, regulation of development, cell death, and hormone signaling (MAP Kinase Group, 2002; Rodriguez et al., 2010).

Plants have a diverse repertoire of hormonal responses involved in plant immune signaling. Plant hormones are critical components in plant defense responses to microbes and insects. Rapid changes to hormone concentrations and production can alter a wide range of plant responses, which are dependent on the specific composition of the hormone response (Walters and Heil, 2007; Pieterse et al., 2012). SA plays an important role in plant disease resistance to pathogens and its biosynthesis is stimulated in response to ETI and PTI (Mishina and Zeier, 2007). Jasmonate biosynthesis induction by wounding is a critical component of plant defense responses to insect attack (Howe et al., 1996). MAPK signal transduction pathways are implicated in the control and transmission of many hormone signaling events. MAPK cascades are implicated in the regulation of plant hormone biosynthesis of SA (Beckers et al., 2009), ethylene (Liu and Zhang, 2004), and JA (Seo et al., 1999; Seo et al., 2007; Wu et al., 2007; Kandoth et al., 2007) during defense responses.

Two of the best-characterized MAPKs are Arabidopsis AtMPK6 and AtMPK3, and their tomato orthologs SlMPK1/2 and SlMPK3, and their Nicotiana orthologs SIPK and WIPK, respectively. Loss of SlMPK1/2, results in reduced resistance to caterpillar herbivores, attenuated JA accumulation in response to wounding, and reduced expression
of the defense gene, proteinase inhibitor II (PI-II; Kandoth et al., 2007). Silencing of AtMPK6 compromised plant defense against virulent and avirulent pathogens, indicating a role of MPK6 in ETI and PTI (Menke et al., 2004). Silencing WIPK, the *AtMPK3* ortholog in tobacco, resulted in reduced accumulation of JA in response to wounding (Seo et al., 1999, 2007). Gain of function studies using tobacco plants expressing constitutively active MAPKKs, which specifically activate SIPK and WIPK, and plants with gain of function activation of SIPK alone, develop cell death phenotypes similar to pathogen induced HR (Jin et al., 2003; Zhang and Klessig, 2001). Silencing of both WIPK and SIPK in tobacco results in decreased JA accumulation in response to wounding, increased SA accumulation, and expression of the pathogenesis related (PR) gene *PRI* (Seo et al., 2007).

**Plant cell death and its involvement in immune responses**

Cell death plays an important role in plant development and resistance to disease. The mechanisms of cell death have been well studied in animal systems. Plants share some similar cell death characteristics, however, there are distinct differences observed between plants and animals. Animals have three types of cell death: apoptosis, autophagy, and necrosis, which are classified based on cell morphology (Kroemer et al., 2009). Apoptosis is characterized by chromatin condensation, nuclear segregation, and reduction of cell volume, which is followed by the fragmentation of the cell into apoptotic bodies. This is associated with the activation of caspases and DNA fragmentation, although these two can also occur during non-apoptotic cell death (van Doorn et al., 2011). Autophagy is the bulk degradation and recycling of cytoplasmic material that involves the formation of autophagosomes that fuse with the vacuole
(plants) or lysosomes (animals). Necrosis is associated with inflammation and is morphologically defined by a gain in cell volume (Kroemer et al., 2009). There are two main reasons why plant cells cannot undergo classic mammalian apoptosis; first, the cell wall prevents the breakdown of the cell into apoptotic bodies, and second, there are no dedicated phagocytic cells in plants. However, plants do have large vacuole systems that are involved in cell death. Lytic vacuoles are involved in the reduction of cell volume, which is followed by the rupturing of the tonoplast and release of vacuolar proteases and nucleases that can destroy the protoplast and other parts of the cell (Hatsugai et al., 2009).

In plants, cell necrosis can be induced by many abiotic stresses, successful recognition of pathogens, and invasion by necrotrophic pathogens. The plant immune system can be separated into two branches. The first utilizes transmembrane pattern recognition receptors (PRRs) that recognize PAMPs. PRRs are receptor-like kinases with an extracellular domain that consists of leucine-rich repeats that are able to perceive PAMPs, and an intracellular kinase domain involved in signal transmission via MAPK cascades. Recognition of PAMPs triggers an immune response known as PAMP-triggered immunity (PTI). The second branch of innate immunity mostly proceeds within the cell and is activated by pathogen effectors delivered into the cytoplasm to suppress PTI. Resistance (R) proteins, encoded by resistance genes, are intracellular receptor proteins that recognize specific effectors. R proteins are characterized by their nucleotide binding site (NB) and leucine-rich repeats (LRR). They have evolved to recognize specific pathogen effector proteins to induce effector-triggered immunity (ETI). If plants lack the proper R gene to match a specific effector, the effector will suppress PTI resulting in effector-triggered susceptibility (Jones and Dangl, 2006). ETI can lead to
disease resistance and a hypersensitive response (HR) at the site of infection. The HR involves an increase in the expression of defense related genes and pathogenesis related (PR) genes, the synthesis of antimicrobial secondary metabolites, and localized cell death at the area of infection (Zurbriggen, 2010; Morel 1997). It is important to note that localized cell death and PR gene induction can occur in response to various abiotic stresses (Mur et al., 2008).

A central part of HR, following the recognition of pathogen effectors, is rapid calcium influx, and the production of reactive oxygen species (ROS) and nitric oxide intermediates (NO) (Zhang et al., 2010). The production of ROS in the apoplast is mediated by NADPH oxidase encoded by the rboh gene family, which contributes to a rapid oxidative burst immediately following recognition of a pathogen. ROS can also be generated as by-products of photosynthesis and respiration in response to pathogen infection (Breusegem and Dat, 2006). There is evidence that Arabidopsis MPK3/MPK6 and tobacco WIPK/SIPK are activated by hydrogen peroxide and superoxide anions (Kovtun et al., 2000; Samuel, et al., 2000; Moon et al., 2003), which indicates ROS acts upstream of MAPK cascades (Pitzschke and Hirt, 2006). However, MAPK and calcium-dependent kinase pathways are also involved in an amplification network upstream of rboh genes (Nakagami et al., 2005; Ren et al., 2002, 2006; Yoshioka et al., 2009; Asai and Yoshioka, 2008). This indicates that MAPKs can be involved both downstream and upstream of ROS production, however, the complex dynamics of the ROS signaling network are not fully understood.
Plant Antioxidants

Since plants cells are subject to oxidative pressure from many different sources, proper balance of ROS production and scavenging is critical for redox homeostasis that can effect plant growth, development, and response to pathogens. Much of the ROS generated in cells is produced by the chloroplast, however, ROS can also be derived from leakage of the mitochondrial electron transport chain and from beta-oxidation in peroxisomes. Plants have developed complex antioxidant systems to counterbalance oxidative stresses. Antioxidants can be divided into two groups: non-enzymatic and enzymatic. Non-enzymatic antioxidants consist of ROS scavengers such as ascorbate, glutathione, and hydrophobic molecules (i.e. tocopherols, carotenoids, xanthophylls) that are able to buffer the cell from ROS stress. Non-enzymatic scavengers work in concert with enzymatic scavengers such as ascorbate peroxidase, superoxide dismutase, and catalase to convert harmful oxygen intermediates into less harmful products (Bhattacharjee, 2012). The antioxidant status of a cell plays an important role in plant defenses, especially in responses to biotic stress and wounding. In the chloroplast and mitochondria, superoxide dismutases rapidly convert singlet oxygen and superoxide anions to hydrogen peroxide. In the chloroplast, hydrogen peroxide can be neutralized by ascorbate peroxidase, and within peroxisomes catalase converts hydrogen peroxide into water and oxygen (Apel and Hirt, 2004). Catalase plays an important role in the regulation of cellular hydrogen peroxide levels and loss of catalase can lead to the accumulation of ROS from photorespiration (Queval et al., 2007).
Methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum*) varieties Rio Grande, Castlemart, and MicroTom, and the wild species *Solanum pimpinellifolium*, were grown in AR66L growth chambers (Percival Scientific, Perry, IA) under a 16-hour light (110±20 µEm⁻²s⁻¹) and 8-hour dark regime. For VIGS, plants were grown at 20°C (day) and 18°C (night) for three weeks following infiltration, then transferred to 27°C (day) and 22°C (night) for one week prior to sampling.

Generation of VIGS Constructs

The modified tobacco rattle virus (TRV) vectors, pTRV1 and pTRV2 (Liu et al., 2002), used for virus-induced gene silencing were obtained from S.P. Kumar-Dinesh (Yale University, New Haven, CT). A portion of each target gene was cloned into the multiple cloning site (MCS) of the pTRV2 vector. A fragment for each target gene, which was predicted *in silico* to have minimal off-target gene silencing, was amplified from a cDNA library generated from Rio Grande. MPK1/2 were co-silenced using a 577 bp fragment PCR-amplified from the MPK1-ORF. Tomato MPK1 and MPK2 are 95% similar at the amino acid level. MPK3 was specifically silenced using either a 406 bp fragment amplified from the 3’ UTR (TS1-VIGS) or a 300 bp fragment amplified from the ORF of MPK3 (TS2-VIGS). The 300bp MPK3 sequence does not have any >21 nt stretches that are identical to the MPK1/2 sequence used for the silencing vector. Forward and reverse primers were designed with flanking restriction sites for ligation into the MCS of pTRV2 (Table 1.1). Ligation products were expressed in One Shot DH5α competent cells (Invitrogen/Life Technologies, Grand Island, NY). Silencing vectors...
were confirmed by sequencing (Eton Biosciences Inc., Durham, NC). Primers used for the generation of target gene fragments are listed in Table 1. The pTRV1 and pTRV2 silencing vectors were transfected into *Agrobacterium tumefaciens* strain GV3101. A single colony was selected and inoculated into a liquid culture supplemented with kanamycin (50 µg/mL), rifampicin (50 µg/mL), and gentamycin (30 µg/mL). The culture was incubated overnight at 30°C. To prepare the infiltration media, the overnight culture was centrifuged at 5,000 g for 10 minutes at 4°C. The pellet was washed twice with 10mM MgCl₂ and resuspended in infiltration medium [10mM MgCl₂, 10mM MES, and 150mM acetosyringone. (3’, 5’-Dimethoxy-4’-hydroxyacetophenone, Acros Organics, New Jersey, USA)] to a total OD₆₀₀ of 0.2 and 0.4 for pTRV2 and pTRV1, respectively. Infiltration medium was forced into the cotyledons of 10-14 day old seedlings using a needleless syringe. Plants were sampled after four weeks.

**Protein Extraction and Immunoblot Analysis**

Plant tissue was flash frozen in liquid nitrogen and subsequently ground in a chilled mortar and pestle. Ground tissue was homogenized with extraction buffer (50 mM HEPES KOH (pH 7.6), 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM NaF, 20% [v/v] glycerol, 0.5% [w/v] polyvinylpyrrolidone, 2 mM DTT, 1 mM PMSF, and 10 µM leupeptin). Protein concentration was determined using Bio-rad Protein Assay (Bio-rad, Hercules, CA), and BSA was used to generate a standard curve. 50 µg of each sample was mixed with SDS gel loading buffer (100 mM Tris pH 8.0, 10 mM EDTA, 50% [v/v] glycerol) and separated via electrophoresis on a 10% polyacrylamide gel. Separated proteins were transferred to immobilon-P PVDF membranes (Millipore, Billerica, MA) using a mini transblot electrophoretic transfer cell
(Bio-rad). The membranes were blocked for one hour in TSB-Tween 20 (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% [v/v] Tween 20) containing 5% BSA (Fraction V, Fisher, Waltham, MA). Membranes were washed twice with TBS-Tween20 then incubated in primary antibody overnight at 4°C. Blots were washed five times in TBS-Tween20 then incubated in alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO). After five washes in TBS-Tween20, blots were incubated for five minutes in LumiPhos (Pierce, Rockford, IL) or Bio-rad chemiluminescence reagent (Bio-rad) and visualized with HyBlot CL autoradiography film (Denville Scientific Inc., Metuchen, NJ).

Primary antibodies used for immunoblotting were anti-p44/p42 MAPK (Erk1/2, Cell Signaling Technology, Danvers, MA) at 1:2500 in TBS-Tween20 with 5% [w/v] BSA. Anti-p44/p42 specifically recognizes active MAPKs phosphorylated on the Thr and Tyr residues within the TEY phosphorylation motif. Anti-AtMPK6 (Sigma-Aldrich) was generated from Arabidopsis MPK6 and is able to detect tomato homologs MPK1/2, and was used at 1:5000 in TBS-Tween20. Anti-Catalase (EMD Millipore, Darmstadt, Germany) was used at 1:2500 in TBS-Tween 20. The secondary antibody, anti-rabbit alkaline phosphatase-conjugated (Sigma-Aldrich), was used at 1:20,000 in TBS-Tween20. Membranes were stained using MemCode Reversible Protein Stain Kit – for PVDF Membranes (Pierce).

**RNA Extraction and Gene Expression Analysis**

For reverse transcription (RT) quantitative real-time PCR (qPCR), total RNA was extracted from tomato leaf tissue (~100 mg) using TRIzol® reagent (Life Technologies, Grand Island, NY, USA). RNA was quantified using a SmartSpec Plus
spectrophotometer (Bio-rad, Hercules, CA, USA) and confirmed by electrophoresis using a 1% agarose gel. Extracted RNA was treated with Turbo DNase (Life Technologies). First strand cDNA was synthesized using 1 µg of RNA with iScript™ Reverse Transcription Supermix for RT-qPCR (Biorad). The protocol for the synthesis of cDNA was: priming (25°C, 5 min), reverse transcription (42°C, 30 min), and RT inactivation (85°C, 5 min). The cDNA was diluted 1:30 for gene expression analysis via qPCR. RT-qPCR was carried out using a Bio-rad C1000 and SsoAdvanced Universal SYBR Green Supermix (Bio-rad). The protocol for RT-qPCR was: 95°C for 30 s, followed by 50 cycles of 95°C for 15 s and 55°C for 30 s. The melting curves were analyzed at 55-95°C for 80 cycles (0.5°C increments for 5 s). Primers used for RT-PCR are listed in Table 2. Primer specificity was confirmed by melting curve analysis for every reaction. Non-reverse transcribed samples were run as a control for each RT-qPCR run. Three technical replicates were used for each sample during every RT-qPCR run. Average C_T and primer efficiencies were determined using Real-time PCR Miner (http://ewindup.info/miner/) (Zhao and Fernald, 2005). Fold difference in target gene expression was calculated using the Pfaffl method (Pfaffl, 2001) and 18s-rRNA or β-actin (ACT2) as the reference gene.

**Oxidative Burst Assay**

Ninety-six leaf disks from *S. lycopersicum* variety Rio Grande were cut using a cork borer with a diameter of 4 mm. One disk was placed abaxial side down into each well of a 96-well plate (Lumitrac 200; Greiner Bio-One, Monroe, NC, USA) and incubated in water overnight (ambient room temperature and light). Leaf disks were excised from control VIGS plants (VIGS-GFP) or from distinct regions of *MPK1/2/3*-triple silenced plants (TS1-VIGS), such as lesion containing area (Lesion) and non-lesion
areas most distant from the lesion (No Lesion). Each 96-well plate consisted of both GFP-VIGS and TS1-VIGS samples with or without Flg22 (GenScript, Piscataway, NJ), to measure the Flg22-induced ROS accumulation or basal ROS levels, respectively.

Immediately before treatment, the water was removed and each well was filled with an aqueous solution containing 34 µg/mL luminol (Sigma–Aldrich) and 20 µg/mL horseradish peroxidase (MP Biomedicals, Santa Ana, CA, USA), and with or without 10 nM flg22. Immediately following treatment, chemiluminescence was measured for 60 min, with readings taken every 64 s, using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) in Kinetic Read Mode, set to read endpoint luminescence in relative luminescence units (RLU). Data were collected using Gen5 Data Analysis Software (BioTek).

**Jasmonic Acid and Salicylic Acid Analysis**

Leaves were excised from *MPK1/2/3*-VIGS plants and sectioned into tissue samples that contained lesions, tissue adjacent to lesions, or tissue that was most distance to the lesions. Control leaves were left untreated or wounded using a hemostat. One hour after wounding, leaves were excised and the midvein was removed. Samples from multiple plants were pooled so that each sample contained ~300 mg of leaf tissue, which was flash frozen in liquid nitrogen for JA and SA analysis. Samples were homogenized in liquid nitrogen using a chilled mortar and pestle. Frozen powder was transferred to a glass culture tube. 100 ng of the JA internal standard, dihydrojasmonic acid (dhJA) and 100 ng of the SA internal standard, 2-hydroxybenzoic acid-d₆ [SA-d₆] (C/D/N Isotopes Inc., Pointe-Claire, Quebec), were added to the tube. The internal standard dhJA was prepared from dihydro-methyl jasmonate (Bedoukian Research, Danbury, CT) as
previously described (Kandoth, 2007). Added to the tube was 0.6 mL of 66% [v/v] 1-propanol and was vortexed for 10 s, followed by 1 mL of dichloromethane and vortexed for 10 s. Samples were centrifuged at 3000 rpm for 5 min in an Allegra X-15R (Beckman Coulter, Pasadena, CA). The lower dichloromethane phase was transferred to a 4 mL screw-top glass vial. Samples were derivatized using trimethylsilyldiazomethane in hexane for 30 min at 30°C. This was neutralized by a subsequent incubation with 2M acetic acid in hexane for 30 min at 30°C. Volatile collection traps were made by sandwiching ~20 mg Super-Q matrix (Alltech Associates, State College, PA) between layers of glass wool in a Pasteur pipette. The trap was connected to the vial through a septa and connected to an air handler with a flow rate of ~500-1000 mL/min. The vial was placed on a heating block at 70°C and supplied with a steady flow of nitrogen gas. Once the vial was completely dry, the vial was transferred to a heating block at 200°C for an additional minute. Volatile compounds were eluted from the trap with 200 µL of dichloromethane.

Volatile eluted from the Super-Q matrix were analyzed by GC-MS using electron impact ionization (EI) in selective ion mode. Briefly, an HP 5890 gas chromatograph equipped with a split/splitless injector (splitless mode, injection volume 1 ml) was interfaced to an VG-70S magnet sector mass spectrometer (Waters Corp, Milford, MA). Compounds were separated on an Restek RTx-5 (30 m in length, 0.25-mm ID, 0.2-mm film) column preheated to 80°C. After injection, the temperature was increased at 10°C/min to 130°C, then 3°C/min to 180°C, and finally 10°C/min to 300°C, and held at 300°C for 10min. Helium was used as the carrier gas at 10 psi. Specific EI conditions were 70 eV, selection monitoring at 4,000 resolution. Retention time for the
SA (152.0473) was 9 min 19 sec, and for the SA-d₆ (156.0725) was 9 min 16 sec. The
retention time for JA (224.1412) was 21 min 19 sec, and for the dhJA (226.1569) 21 min
28 sec.

**SEM and TEM Sample Preparation and Imaging**

For SEM sample preparation, leaf tissue from GFP-VIGS and *MPK1/2/3*-VIGS
plants were excised and fixed in 100 mM cacodylate buffered (pH 7.2) 2.5%
glutaraldehyde for 3-4 hours at room temperature. Samples were washed several times in
100 mM cacodylate buffer (pH 7.2). The tissue was post-fixed in 100 mM cacodylate
buffered (pH 7.2) 1% osmium tetroxide for 1 hour at 4°C, followed by 3 washes in 100
mM cacodylate buffer (pH 7.2). Sample was dehydrated in a series of 50% to 95%
ethanol washes, and twice in 100% ethanol. Samples were dried, coated with gold, and
mounted. Samples were imaged using a Vega 3 SEM (TESCAN, Czech Republic).

For TEM sample preparation, leaf tissue GFP-VIGS and *MPK1/2/3*-VIGS plants
were excised and fixed in 100 mM cacodylate buffered (pH 7.4) 2.5% glutaraldehyde
overnight at room temperature. Samples were washed several times in 100 mM
cacodylate buffer (pH 7.4). The tissue was post-fixed in 100 mM cacodylate buffered (pH
7.4) 1% osmium tetroxide for 1 hour at 4°C, followed by 3 washes in 100 mM cacodylate
buffer (pH 7.4). The sample was dehydrated in a series of 50% to 95% ethanol washes,
and twice in 100% ethanol. The tissue was incubated in acetone then embedded in
EMBed 812 (Electron Microscopy Sciences, Hatfield, PA). Samples were imaged using
an H8000 TEM (Hitachi, Tokyo, Japan).
Table 1.1: Sequences of primers used for pTRV2 vector construction

<table>
<thead>
<tr>
<th>Target</th>
<th>Accession</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPK1/2</td>
<td>MPK1-AY261512 MPK2-AY261513</td>
<td>5'-GGCGCGAGCTCCATGGTGGCAGGGTTCTATTCC-3' (SacI)</td>
<td>5'-CGGCCTCGAGGCCTAGGTCACGATACT-3' (XhoI)</td>
<td>577</td>
</tr>
<tr>
<td>MPK3-3'UTR</td>
<td>AY261514</td>
<td>5'-GGCGCGCTAGAGCATAAAGAGGAATACATG-3' (XbaI)</td>
<td>5'-CGCGCGGATCCACACCAGAATGAAATGC-3' (BamHI)</td>
<td>406</td>
</tr>
<tr>
<td>MPK3-ORF</td>
<td>AY261514</td>
<td>5'-GGCGCGTCTAGAGGTTTGAGCTAACATG-3' (XbaI)</td>
<td>5'-CGCGCGGATCCTACGGATTGCTAATAC-3' (BamHI)</td>
<td>299</td>
</tr>
</tbody>
</table>

Table 1.2: Sequences of primers used for RT-qPCR gene expression analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Accession</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPK1</td>
<td>AY261512</td>
<td>5'-GCTGACAGATGGTTGAGCTAC-3'</td>
<td>5'-TCCACCCCATTTGATACATCA-3'</td>
<td>176</td>
</tr>
<tr>
<td>MPK2</td>
<td>AY261513</td>
<td>5'-TACTCGCTCGTTGGCTGAGGCAATG-3'</td>
<td>5'-TTGGAGTACAGGGAAAACATGG-3'</td>
<td>171</td>
</tr>
<tr>
<td>MPK3</td>
<td>AY261514</td>
<td>5'-GCAACTCCCAACATCCAC-3'</td>
<td>5'-TCTGCTCCTTCCTCCTATCCCT-3'</td>
<td>233</td>
</tr>
<tr>
<td>PI-II</td>
<td>X94946</td>
<td>5'-ATTATCCATTGTTGCTGGCTAC-3'</td>
<td>5'-CCTTTTGGATTCATTCTGCTAC-3'</td>
<td>198</td>
</tr>
<tr>
<td>PR-1</td>
<td>Y08804</td>
<td>5'-GGATCGGACCACTGCTTTCTCCTAC-3'</td>
<td>5'-GCAACCTTTAAAAAAGGGAATAAT-3'</td>
<td>193</td>
</tr>
<tr>
<td>ICS1</td>
<td>NM001247865</td>
<td>5'-TCCAGGCTTGAGGTTGATGAG-3'</td>
<td>5'-ACCAACAGGCCAATGACATA-3'</td>
<td>143</td>
</tr>
<tr>
<td>ACT2</td>
<td>XM004249818</td>
<td>5'-AGATGGGCTATGAAGAAGG-3'</td>
<td>5'-ACAACAGGAGCTTCTAGAG-3'</td>
<td>165</td>
</tr>
<tr>
<td>18S-rRNA</td>
<td>X51576.1</td>
<td>5'-GTCCAGACATAAGGATTGAA-3'</td>
<td>5'-TAACCCAGACAAAATGTCGCGTCA-3'</td>
<td>101</td>
</tr>
</tbody>
</table>
Results

Spontaneous lesion formation in MPK1/2/3-silenced plants

MAPKs MPK1, MPK2, and MPK3 were co-silenced in Solanum lycopersicum variety Rio Grande using two virus-induced gene silencing (VIGS) constructs, TS1-VIGS and TS2-VIGS (TS stands for ‘triple silencing construct’, Figure 1.1A). Both TS1-VIGS and TS2-VIGS targeted the same 577 bp sequence of the ORF of MPK1/2, however, TS1-VIGS targeted a 406 bp region of the 3’-UTR of MPK3 and TS2-VIGS targeted a 300 bp sequence of the ORF of MPK3. As a control, a portion of GFP was cloned into a pTRV2-silencing vector (Control VIGS). Seedlings were infiltrated with Agrobacterium-containing either pTRV2-TS1 or pTRV2-TS2 (Figure 1.1B). TS1-VIGS plants develop necrotic lesions gradually between 21 and 28 days following infiltration, while TS2-VIGS plants develop lesions rapidly between 14 and 21 days after infiltration. Lesions typically start at the tip of a leaflet, although they can develop on any of the green, aerial portions of the plant, and rapidly develop into uncontrolled and widespread cell death (Figure 1.1C). TS1 plants have a slightly stunted and TS2 plants a severely stunted growth phenotype. The severity of the TS2 growth phenotype is most likely due to the development of lesions on the stems and petioles (Figure 1.1D and 1.1E), which can cause whole leaves or the entire plant to die. TS2 plants are likely more stunted than TS1, due to the earlier development of lesions on their stems leading to reduced or blocked nutrient and water transport. When infiltrated with TS2-VIGS, similar lesion phenotypes were observed in two other varieties of tomato, Castlemart (Figure 1.1F) and MicroTom (Fig 1G), and the wild species of tomato, S. pimpinellifolium (Figure 1.1H).
Figure 1.1: Spontaneous lesions in MPK1/2/3-VIGS plants
A) Diagram of MPK1/2 and MPK3 target sequences used for pTRV2 silencing vectors. TS1-VIGS uses a 577 bp fragment amplified from the ORF of MPK1 and a 406 bp fragment from the 3′-UTR of MPK3. TS2-VIGS uses the same MPK1/2 fragment, but uses a 300 bp fragment from the ORF of MPK3.

B) S. lycopersicum variety Rio Grande plants infiltrated with Agrobacterium containing either pTRV2-GFP (control VIGS), or pTRV2 containing one of the two constructs shown in (A) (TS1-VIGS) or (TS2-VIGS). Lesions develop on the leaves (C), stems (D), and petioles (E) of Rio Grande TS1-VIGS plants. In S. lycopersicum varieties Castlemart (F) and MicroTom (G) and in the wild tomato species S. pimpinellifolium (H) TS2-VIGS plants develop spontaneous lesions on
leaves four weeks after *Agrobacterium* infiltration with TS2-VIGS (five or more weeks for *S. pimpinellifolium* plants). All images were taken between four and five weeks following infiltration.

To confirm lesion formation is not due to bacteria or fungal infection, control VIGS leaf tissue and TS1-VIGS lesion-containing leaf tissue were observed using scanning electron (SEM, Figures 1.2A and 1.2B) and transmission electron microscopy (TEM, Figures 1.2C-F). In areas with lesions, TS1 plants had completely collapsed cells, trichomes, and stomata; which formed a distinct boarder between living and dead cells (Figure 1.2B) as compared to the healthy, turgid cells of control VIGS samples (Figure 1.2A). TEM revealed that the TS1-VIGS samples showed hallmarks of hypersensitive response-like cell death with some features of vacuolar cell death (Doorn et al., 2011), such as disintegration of organellar membranes, separation and disruption of thylakoid grana, vacuolarization, and separation from the cell wall and cleavage of the plasma membrane (Figures 1.2D-F). Together, SEM and TEM analysis of TS1 plants reveals that the lesions are developing spontaneously, show features of the HR, and are not due to a bacterial or fungal infection.

**MPK1/2/3 silencing in TS1- and TS2-VIGS plants**

To better understand the molecular basis of spontaneous lesion formation, *MPK1/2/3* expression and MPK1/2 proteins levels were determined in TS1- and TS2-VIGS silenced plants. The commercial anti-AtMPK6 antibody, raised against a peptide sequence from the *Arabidopsis* homolog of tomato MPK1/2, is able to recognize a 48 kDa band that corresponds to tomato MPK1/2. Currently, there are no reliable commercial antibodies for the detection of tomato MPK3. Examining individual leaves with lesions from TS1-VIGS plants, we found that MPK1/2 protein levels tended to vary
Figure 1.2: Characteristics of cell death in lesion areas of *MPK1/2/3*-silenced plants. Scanning electron microscopy imaging of control VIGS (GFP-VIGS) plants (A) and lesion areas of TS1-VIGS plants (B). Transmission electron microscopy imaging of control VIGS (C) and TS1-VIGS plants (D-F). Tr = trichome, St = stomata, Ch = chloroplast, Th = thylakoid stack, SG = starch granule. Plants were imaged 4-5 weeks.
following infiltration of Agrobacterium containing the VIGS silencing vectors. Black arrowheads indicate areas of vacuolization. White arrow indicates separation and disintegration of the plasma membrane. Images are representative of multiple biological replicates. (SEM and TEM images courtesy of Dr. Erika Balogh, unpublished results).

between areas on the same leaf and between leaves of the same plant. Some variability in gene silencing efficiency is typical for VIGS (Stratmann and Hind, 2011). Although MPK1/2 levels were variable, there were certain trends common in all samples. The strongest MPK1/2 silencing always correlated with lesion areas (Figures 1.3A and 1.3B), which was associated with the accumulation of unknown proteins at ~55 kDa and ~40 kDa. MPK1/2 silencing variability was further examined in greater detail by sectioning lesion-containing leaves and measuring MPK1/2 protein levels. Again, areas with the strongest MPK1/2 silencing were associated with lesions and accumulation of the 55 kDa and 40 kDa proteins (Figure 1.3B, upper panel). Some samples had reduced MPK1/2 protein levels only in lesion areas, but normal MPK1/2 levels in all other areas of the leaf (Figure 1.3B, lower panel). Similarly, TS2-VIGS plants also demonstrated variable MPK1/2 protein levels (Figure 1.3C). The observed decrease in MPK1/2 protein levels were correlated with decreased kinase activity in response to Flg22 (Figure 1.3D). Leaf disks taken from control and TS1-VIGS plants were floated on water then treated with 10 nM Flg22. Leaf tissue containing lesions and areas adjacent to lesions showed significant decrease in phosphorylated MPK1/2, which indicates reduced kinase activity. In TS1- and TS2-VIGS plants MPK1/2 expression levels were equally reduced in both lesion and non-lesion areas compared to control plants (Figure 1.3E). The level of MPK3 expression was only determined for lesion areas of TS2-VIGS plants and was found to be significantly more reduced in TS2-VIGS plants compared to control plants than TS1-VIGS plants compared to control plants.
Figure 1.3: MPK1/2 protein levels and expression levels of MPK1, MPK2, and MPK3 in TS1-VIGS and TS2-VIGS plants. Total protein was extracted and 50 μg samples were separated using SDS-PAGE and subjected to immunoblot analysis using anti-AtMPK6 antibody to determine MPK1/2 levels (A-C). A) Tomato leaves from plants infiltrated with either pTRV2-GFP (Control VIGS) or pTRV2-MPK1/2/3-1 (TS1-VIGS) were sectioned according to the diagram; separated into three sections: the tip of the leaf (T; location of the lesion on the TS1-VIGS leaves), the midsection (M), and the area of the leaf closest to the petiole (P). The upper band (~55 kDa) and lower band (~48 kDa)
represent CAT1 and MPK1/2, respectively. Immunoblots stained with coomassie to show equal protein loading (CBB) B) Upper and lower panels show immunoblots of leaves from different TS1-VIGS plants, sectioned according to the diagram. Each panel (A and B) represents one individual leaf and each lane represents a section from that leaf. Immunoblots are representative of multiple biological replicates. C) Total protein content from plants infiltrated with pTRV2-MPK1/2/3-2 (TS2-VIGS) analyzed by immunoblot. The roman numerals represent one leaf from different individual plants. D) Leaf disks taken from control or TS1-VIGS plants were treated with 10 nM Flg22 for 15 min. Total protein content was analyzed by immunoblot and phosphorylated MPK1/2 detected by anti-pERK antibody. E) Expression levels of MPK1, MPK2, and MPK3 were determined by RT-PCR normalized to the reference gene, β-actin. Bars represent the fold expression difference between TS1-VIGS (black) and TS2-VIGS (grey) to the control VIGS (white). MPK1/2 expression levels represent the mean of 6 and 5 biological replicates from TS1-VIGS and TS2-VIGS plants, respectively. MPK3 expression levels represent the mean of 4 and 2 biological replicates from TS1-VIGS and TS2-VIGS plants, respectively. Error bars represent the standard deviation of the mean. * p < 0.05 TS1-VIGS vs. TS2-VIGS, MPK3 expression, ** p < 0.05 MPK1, MPK2, and MPK3 expression compared to Control VIGS (student t-test).

**Accumulation of catalase in MPK1/2/3-silenced lesion tissue**

The 55 kDa protein that accumulated in lesion areas was identified as Catalase1 (tomato CAT1) by mass spectroscopy (Hind and Stratmann, unpublished). There are three catalase isozymes in tomato, however, only CAT1 was found to be highly expressed in the lesion areas. To further confirm catalase accumulation, tomato leaf samples from control VIGS plants and TS1-VIGS plants with no lesions, some lesions, or many lesions were analyzed via immunoblot and probed with anti-catalase antibody. In control plants there was no detectable catalase, however, TS1-VIGS samples showed a correlation between the abundance of catalase and the amount and severity of lesions. Sequence alignment revealed that six out of 12 amino acids are shared between CAT1 and the Arabidopsis MPK6 peptide used to generate anti-AtMPK6 antibody, which explains why catalase was detected by anti-AtMPK6 by immunoblot.
To determine if increased catalase protein accumulation is associated with increased catalase activity, tomato leaf samples were analyzed via zymogram using hydrogen peroxide as an artificial substrate. Samples were first separated using native gel electrophoresis and probed with anti-AtMPK6, which showed greater accumulation of catalase in lesion-containing samples. Similar samples were subjected to a catalase zymogram assay. TS1-VIGS samples with lesions showed increased catalase activity compared to non-lesion TS1-VIGS and control VIGS samples. The increased catalase activity directly corresponds to areas of increased catalase protein accumulation detected by immunoblot.

**Reactive oxygen species levels in MPK1/2/3-silenced plants**

Previous studies have shown that high concentrations of hydrogen peroxide strongly induce catalase genes, indicating a direct signaling role of H$_2$O$_2$ in the regulation of antioxidant gene responses (Guan and Scandalios, 2000; Polidoros and Scandalios, 2002). Since lesion areas of MPK1/2/3-silenced plants are associated with accumulation and increased activity of catalase, we examined this tissue for altered ROS levels. Samples from TS1-VIGS and control VIGS were stained with 3,3’-diaminobenzidine (DAB), which is oxidized by H$_2$O$_2$ in the presence of peroxidases to produce a reddish-brown stain (Thordal-Christensen et al., 1997). No detectable DAB staining was observed in TS1-VIGS plants (data not shown), indicating that H$_2$O$_2$ levels are either unaffected in MPK1/2/3-silenced plants, DAB staining is not sensitive enough to detect small H$_2$O$_2$ increases, or there is accumulation of a different ROS, such as superoxide anions (O$_2^{•−}$) that could be causing the cell death.
Using an alternative approach, ROS levels were measured using a luminol-based chemiluminescence assay, which is able to detect accumulation of both H$_2$O$_2$ and O$_2$$^-$$^-$ levels. This assay uses horseradish peroxidase (HRP) as a necessary enzyme to catalyze the first one-electron oxidation step of the luminol reaction (Niederländer et al., 2008). Measuring background levels of ROS in control VIGS and TS1-VIGS plants showed no detectable ROS levels (data not shown), which indicates that there are no differences in basal ROS levels or that this assay is unable to measure background ROS levels. In order to rapidly induce ROS levels, leaf disk samples were treated with the bacterial elicitor flg22, which is known to rapidly induce an oxidative burst (Boller and Felix, 2009). Leaf disks were excised from control VIGS and TS1-VIGS leaves with (L) and without lesions (NL), incubated with luminol, HRP, and 10 nM flg22. ROS accumulation was immediately measured using a plate reader. Unexpectedly, ROS levels were much higher in TS1-VIGS non-lesion leaf disks compared to control VIGS (GFP-VIGS) leaf disks. Interestingly, leaf disks from TS1-VIGS lesion areas had an even greater accumulation of ROS than control plants and the levels were significantly greater than TS1-VIGS non-lesion leaf disks (Figure 1.5A).

Rapid generation of ROS, specifically superoxide anion and hydrogen peroxide, is one of the early steps in pathogen-triggered hypersensitive responses. This oxidative burst is dependent on membrane-bound NADPH oxidase encoded by \textit{rbohD} genes. Mutations in \textit{rbohD} result in elimination of the majority of ROS production in response to pathogens, causing reduced cell death and increased pathogen infection (Torres et al., 2002). NADPH oxidase can also be inhibited by diphenyleneiodonium, which specifically inhibits flavoenzymes, and prevents accumulation of ROS (Foreman et al.,
To determine if the increases in ROS accumulation in the lesion areas of TS1-VIGS plants is dependent on NADPH oxidase, TS1-VIGS lesion leaf disks were treated as described previously and treated with DPI. ROS accumulation was significantly reduced in samples combined with DPI compared to leaf disks without the inhibitor (Figure 1.5B). Together, this indicates that areas with lesions rapidly initiate a very strong NADPH oxidase-dependent oxidative burst, despite very high levels of catalase.

**SA/JA hormone levels in MPK1/2/3-silenced plants**

MAPK signaling is involved in the regulation of plant defense hormone biosynthesis and is also implicated in the transduction of perceived JA and SA signals. *MPK1/2/3*-silenced plants were analyzed for altered JA and SA accumulation one hour after mechanical wounding. JA accumulation peaks between one and two hours in response to wounding (Conconi et al., 1996, Glauser et al., 2008). In unwounded plants, JA levels are close to the detection limit in all plants. Within one hour after wounding, JA rapidly accumulates to high levels in control plants (GFP-VIGS), but not in TS1- and TS2-VIGS plants. TS2-VIGS plants, which develop more severe cell death phenotypes and have strong MPK3 silencing, show no induction or stimulation of JA production or accumulation in response to wounding (Figure 1.6A). Unwounded or wounded control plants exhibit low background levels of SA, however, there is significant SA accumulation in TS1- and TS2-VIGS plants in the absence of a stimulus. Also, TS2-VIGS plants have significantly greater accumulation of SA compared to control plants than TS1-VIGS plants (Figure 1.6B). Previous studies indicate that SA is induced in response to perception of pathogens and not during mechanical wounding (Malamy et al., 1990; Gaffney et al., 1993). Similarly, we observe equal accumulation of SA in each set
Figure 1.4: Increased ROS accumulation in MPK1/2/3-silenced plants after induction of an oxidative burst by flg22. Leaf disks taken from Control VIGS and TS1-VIGS leaves without lesions (TS NL) and with lesions (TS L) were floated overnight on water, then the water was replaced with reaction mix containing 10 nM flg22 alone (A) or 10 nM flg22 plus 10 μM DPI (B). Leaf disks were analyzed for the accumulation of reactive oxygen species using a luminol-based chemiluminescence assay. Luminescence was measured in 64 s intervals. Data represents the mean of four individual leaves with two replicates each at the times indicated ± standard error of the mean. * Significant difference (p > 0.05) in luminescence between TS NL or TS L vs. GFP (A), and TS L + flg22 and TS L + flg22/DPI (B). Results in (A) are representative of 4 biological replicates.
Figure 1.5: Decreased JA accumulation and increased SA accumulation in MPK1/2/3-silenced plants. Control VIGS (GFP-VIGS), TS1-VIGS, and TS2-VIGS plants were sampled before wounding (Unwounded) and 60 minutes after wounding with hemostats (Wounded). 300 mg of leaf tissue was subjected to methylene chloride extraction, derivatization, and vapor phase extraction using a Super Q column to trap all volatile compounds. Samples were analyzed for JA content (A) and SA content (B) by gas chromatography coupled with mass spectroscopy (GC-MS). C) RT-qPCR analysis of PR1a expression in TS1-VIGS and TS2-VIGS lesion areas compared to control VIGS (Fold Expression = 1). Data represents the mean ± standard deviation of two independent replicates. * Indicates significant difference (p > 0.05, student t-test) between TS1- and TS2-VIGS and control VIGS.
of unwounded and wounded plants, indicating that wounding does not increase the accumulation of SA in \( MPK1/2/3 \)-silenced plants.

Pathogenesis-related (PR) genes are strongly expressed during SAR and have been shown to be a marker gene for SA accumulation (Yalpani et al., 1991). In TS1-VIGS, and especially TS2-VIGS plants, PR1a expression was significantly higher in unwounded and wounded samples compared to control plants (Figure 1.6C). Taken together, this indicates that \( MPK1/2/3 \)-silencing causes reduced JA accumulation, increased SA accumulation, and increased expression of an SA-responsive gene in areas with the strongest MPK1, MPK2, and MPK3 reduction and in lesion areas.

**Discussion**

Plants can be exploited by a number of organisms and it is critical that plants put up defenses to reduce the extent of damage or spread of infection. The recognition and relay of external stimuli is required for the induction of defense responses and plants must be able to distinguish the type of stress and initiate the appropriate responses. MAP kinase phosphorylation pathways play important roles in the transduction of stress into cellular responses, especially in defense responses to pathogens and insect herbivory. We found that co-silencing tomato \( MPK1, MPK2, \) and \( MPK3 \) using virus-induced gene silencing causes development of spontaneous, runaway lesions on the green portions of the plant. Lesions only develop on plants where all three MAPKs are silenced and not in plants where they are singly silenced or in MPK1/2-silenced plants (data not shown and Kandoth et al., 2007). Silencing tobacco homologs of tomato \( MPK1/2 \) and \( MPK3, SIPK \) and \( WIPK \), causes decreased accumulation of JA, increased SA levels, and increased
PR1a expression after wounding (Seo et al., 2007). While these findings are similar to ours, we also found that MPK1/2/3-silencing induces the formation of lesions with accumulation of catalase and a heightened sensitivity to flg22. Furthermore, we observed accumulation of SA and increased PR1a expression in both wounded and unwounded plants, whereas Seo et al., 2007, observed these effects only after wounding.

Using VIGS enabled us to observe the effects of MAPK silencing at the seedling stage. In Arabidopsis, mpk6/mpk3 double-null mutants are embryo lethal (Wang et al., 2007), indicating that these MAPKs are required for vital developmental functions. VIGS generally results in reduced transcript levels of target genes, which can be an advantage and disadvantage at the same time. In addition, VIGS may not equally silence target genes throughout the entire plant (Figures 1.3A-C). Variable silencing has been visualized in phytoene desaturase (PDS)-VIGS silenced plants. VIGS of PDS causes mosaic-like photobleaching symptoms. On the other hand, PDS was also silenced in green parts of PDS-VIGS leaves, indicating that the mosaic-like phenotype is more due to the intensity of silencing rather than the presence or absence of silencing (Ruiz et al., 1998; Stratmann and Hind, 2011). If MPK1/2/3-silencing is also mosaic-like, then the spontaneous nature of lesion formation may be due to the initiation of lesions in areas where MPK1/2/3 are strongly silenced resulting in very low MPK1/2/3 protein levels. This hypothesis would indicate that together MPK1, MPK2, and MPK3 negatively regulate some aspect of cell death. This hypothesis is further supported by the severity of the cell death phenotype where MPK3 is better silenced in TS2-VIGS versus TS1-VIGS plants. Alternatively, the cell death phenotype may be a cellular response to the complete disruption of cellular signal transduction pathways. MAPKs are central signaling hubs
involved in the transduction of many stimuli, therefore, both the overexpression as well as the silencing of MAPKs results in cellular chaos, which may induce cell death.

Microscopically and macroscopically, the lesions are similar in appearance to cell death induced by an avirulent-triggered hypersensitive response (ETI). SEM imaging of the hypersensitive response in *N. edwardsonii* induced by tobacco mosaic virus, shows distinct areas where the lesion boarders against healthy cells (Wright et al., 2000), similar to Figure 1.2B. Prolonged activation of *SIPK*/*Ntf4*/*WIPK* by constitutively active MEK2 in tobacco leads to chloroplast disruption and damage (Liu et al., 2007), similar to damage observed in *MPK1/2/3*-silenced plants. The lesions are also very similar in appearance to lesions formed in *lesion simulating disease1* (*lsd1*) mutants (Aviv et al., 2002).

Interestingly, a recent transcriptomics study in *Arabidopsis* that examined global transcriptional changes in *mpk3, mpk6,* and *mpk4* single mutants found novel roles of MPK3 in defense responses (Frey et al., 2014). The results indicate that MPK3 has a role in the negative regulation of inducible defenses. In *mpk3* mutant plants exposed to flg22, there is a significant increase in expression of three genes involved in SA biosynthesis (SID2) and SA signaling (EDS1, PAD4), which suggests MPK3 negatively regulates these defense genes in response to flg22. Although the increase in gene expression was only in response to flg22, these results are similar to the significant increase in SA accumulation we observed in *MPK1/2/3*-silenced tomato plants; where the increase in SA accumulation seems to be dependent of the level of MPK3 silencing (Figures 1.3E and 1.6B). Thus, the stronger MPK3 is silenced, the less SA biosynthesis is negatively regulated and the greater the SA accumulation is in those areas.
MPK1, MPK2 and MPK3 co-silenced via VIGS in *S. pimpinellifolium* have altered abscisic acid-mediated drought tolerance, however, the authors did not report any lesion formation (Li et al., 2013). We observed that VIGS in *S. pimpinellifolium* produced a mild cell death phenotype that takes longer to develop than in *S. lycopersicum* varieties (Figure 1.1).

Development of lesions is correlated with areas exhibiting the strongest *MPK1/2/3* silencing and highest accumulation of catalase protein levels. These areas also have reduced JA accumulation in response to wounding and increased SA accumulation in both wounded and unwounded plants associated with very high PR1a expression. JA accumulation in response to wounding is reduced in *MPK1/2*-silenced tomato plants (Kandoth et al., 2007) and SIPK is required for SA biosynthesis in tobacco (Samuel et al., 2005). In contrast, we show that SA significantly accumulates in *MPK1/2/3*-silenced tomato plants. SA may have an active role in lesion formation, which is well known for the pathogen-induced HR (Yan and Dong, 2014). However, it cannot be excluded that the formation of lesions is due to high, local accumulation of SA, which is toxic to plant cells and is also associated with senescence, another cell death phenomenon (Zhang et al., 2013). Therefore, the SA accumulation may be a consequence of the cell death, or may be the causative factor in lesion development.

There are a number of lesion-mimic mutants that develop lesions spontaneously. One of the best-characterized lesion mimic mutants in *Arabidopsis* is lesion simulating disease1 (*lsd1*). LSD1 is a zinc finger protein that has three zinc finger domains required for association with LSD1-interacting proteins and is a negative regulator of cell death (Li et al., 2013). *lsd1* mutants develop spontaneous cell death lesions triggered by ROS
and SA, which typically develop during long photoperiods or low titer avirulent pathogen infections (Dietrich et al., 1997; Jabs et al., 1996; Kliebenstein et al., 1999; Aviv et al., 2002). LSD1 can interact with a positive regulator of cell death, metacaspase1 (AtMC1), which has caspase-like activity that is important for superoxide-dependent cell death and HR (Coll et al., 2010). LSD1-interacting proteins are present in the cytoplasm, peroxisome, nucleus and plasma membrane, suggesting that LSD1 may act as a housekeeping gene or sensor to monitor ROS levels and regulate cell death in response to changing ROS levels (Mateo et al., 2004). Runaway cell death in lsd1 mutants can be rescued by blocking SA accumulation (Li et al., 2013). This indicates that SA promotes cell death, which is consistent with our findings that SA accumulates in lesion areas of MPK1/2/3-VIGS plants.

Whereas we found high levels of catalase associated with lesions in MPK1/2/3-VIGS tomato plants, in the Arabidopsis lsd1 mutants, peroxisomal CAT levels were reduced (Mateo, 2004). Additionally, LSD1 can physically and genetically interact catalases in Arabidopsis (Li et al., 2013). Interaction between CAT and LSD1 occurs during light-dependent runaway cell death and the cell death phenotype was enhanced in plants co-silenced for catalase and LSD1. While these studies suggest a negative role for catalase in cell death, possibly through detoxification of hydrogen peroxide, we see enhanced catalase expression, protein accumulation, and enzymatic activity in lesions of MPK1/2/3-silenced plants, indicating a positive role for catalase in cell death. It will be important to further explore the relationship between MAPKs, LSD1, and catalase.

One of the early reports on lsd1 found that superoxide accumulation was necessary and sufficient to initiate lesion formation (Jabs et al., 1996). Superoxide
accumulates before initiation and at the leading edge of the lesion as it spreads to healthy cells adjacent to the lesion. Based on this, there is a possibility that the increased ROS accumulation we observe in lesions areas in MPK1/2/3-silenced plants in response to flg22 may be due to high superoxide levels in and around the lesions with reduced levels in areas further from the lesion as reflected in the oxidative burst assay (Figure 1.5A). The accumulation of superoxide can be determined using nitrotetrazolium blue (NTB) staining. We previously tested for ROS accumulation using DAB staining, which is suitable for determining H$_2$O$_2$ levels, but is insufficient for measuring superoxide levels (Jambunathan, 2010).

In Arabidopsis, catalase has also been implicated in lesion formation. Knockout of CAT2 caused lesion formation in a daylength- and photorespiratory-dependent manner due to the hyper-accumulation of ROS (Queval et al., 2007; Chaouch et al., 2010). Catalase has also been suggested to be involved with MAPK signaling pathways. ABA-mediated CAT1 expression was suppressed in an mpk6 mutant and was enhanced in MPK6-overexpression lines in Arabidopsis (Xing et al., 2008). Although we observe the opposite effects, it is possible that silencing of MPK1, MPK2, and MPK3 in tomato has a different effect in the interaction between catalases and MAPKs, which leads to catalase-mediated cell death. In animal systems, catalase can be phosphorylated in response to oxidative stress (Cao et al., 2003). The importance of this phosphorylation was demonstrated in cells that lacked two kinases responsible for catalase phosphorylation, which exhibited increased H$_2$O$_2$-induced apoptosis. This is just one example of catalase regulation by phosphorylation and may explain why the reduction of MPK1/2/3 leads to increased CAT1 expression and CAT1 accumulation. At this point, it can not be ruled out
that any of the MAPKs directly interact with catalase or regulate catalase through phosphorylation. The tomato CAT1 sequence contains a conserved SP motif, the minimal phosphorylation motif for MAPKs, and a conserved MAPK docking motif required for interactions between MAPKs and their substrates. Catalase has also been shown to be a salicylic acid binding protein (Chen et al., 1993a and 1993b; Sanchez-Casas and Klessig, 1994), along with other enzymes such as ascorbate peroxidase, aconitase, and lipoxidase (Ruffer et al., 1995). There is evidence both supporting and against catalase as an SA-interacting protein. This may be an additional layer of complexity involved in the formation of lesions in MPK1/2/3-silenced plants.

Autophagy is another mechanism involved in regulating HR-induced cell death pathways. Autophagy is the bulk degradation of cellular components during stress or starvation, which is required for the recycling and remobilization of nutrients. Autophagy plays both a pro-survival and a pro-death role during HR-induced cell death. In atg mutants, autophagy is implicated in restricting the spread of cell death induced by HR (Li et al., 2005), but also promotes HR-induced programmed cell death (Hofius et al., 2009). Although autophagy and MAPKs are both involved in aspects of HR cell death, there is little evidence that suggests autophagy is regulated by MAPK signaling. Further testing is necessary to determine if the cell death phenotype of MPK1/2/3-silenced plants is due to misregulation of autophagy-mediated cell death.

Silencing of MPK6/SIPK/MPK1/2 and MPK3/WIPK causes altered SA and JA signaling and increased PR1a expression in response to wounding, but not in untreated plants (Seo et al., 2007). In we observed significant accumulation of SA and increased expression of PR1a in both unwounded and wounded MPK1/2/3-VIGS plants.
Constitutive activation of WIPK and SIPK by the tobacco MEK2 (MAPKK) causes the induction of hypersensitive cell death in the absence of a pathogen (Yang et al, 2001). On the other hand, we observe that silencing of the WIPK/SIPK orthologs in tomato causes a similar cell death phenotype. This indicates that a constitutive alteration of the MAPK signaling (silencing or overexpression) renders the cell unable to properly respond to the environment. As a consequence, the cells undergo programmed cell death. Alternatively, this may suggest that MAPK signaling is involved in both the negative regulation and the promotion of cell death. Similar to this, both the overexpression and the silencing of SIPK renders tobacco plants sensitive to ozone (Samuel and Ellis, 2002).
Chapter 2: Identification of a MAP kinase-containing multi-protein complex
Abstract

Mitogen-activated protein kinase (MAPK) cascades are crucial components of signal transduction pathways, which are involved in the transmission of extracellular stimuli into a wide range of cellular responses. These protein phosphorylation cascades consist of three functionally linked kinases: MAPK kinase kinase, MAPK kinase, and MAPK. In plants, MAPK activation by phosphorylation is well understood, but little is known regarding MAPK inactivation by dephosphorylation via MAPK phosphatases. MAPK inactivation plays a role in determining the amplitude and timing of MAPK signaling and activity, which is likely to contribute to signaling specificity. We have identified a MAP kinase-containing protein complex in Arabidopsis thaliana, tomato, and tobacco. Only inactive MAP kinases associate with this complex, unless a phosphatase inhibitor blocks dephosphorylation. After treatment with cantharidin, active MAPKs associate with the protein complex, indicating a possible relationship and cooperation between the MAPK complex and MAPK-inactivating phosphatases. Currently, there is no prior evidence of a multi-protein MAP kinase-containing complex in plants, however, such complexes are common in animal and yeast systems. Determining the composition of MAPK complexes and their association with MAPK phosphatases will advance our understanding of the regulation of MAPKs, which is crucial for how plants respond to the environment.

Introduction

Arabidopsis MPK6 and MPK3 are both involved in the response to diverse environmental stimuli, which induce stimulus-specific output responses (Nakagami, 2005). MAP kinase signaling pathways are conserved in all eukaryotes and are involved
in a range of cellular functions such as plant development and defense responses. The best characterization of MAPKs exists for the model plant, *Arabidopsis thaliana*. The *Arabidopsis* genome contains ~80 putative MAPKKKs, 10 MAPKKs, and 20 MAPKs (MAP Kinase Group, 2002; Andreasson and Ellis, 2010). The discrepancy in number of MAPKKs to MAPKs indicates that MAPKKs are able to phosphorylate multiple MAPKs. For example, in *Arabidopsis*, MKK1 phosphorylates and activates MPK3 in response to salt stress (Kim et al., 2011), MPK4 in response to wounding, drought, hydrogen peroxide, and bacterial elicitors (Qiu et al., 2008; Teige et al., 2004; Pitzschke et al., 2009; Gao et al., 2008), and MPK6 as a key step during ABA-dependent H₂O₂ production (Xing et al., 2008). Additionally, a single MAPK can be phosphorylated by multiple MAPKKs. For example, MPK6 is phosphorylated by MKK2 during salt stress (Teige et al., 2004), by MKK4/5 in response to flg22 (Asai et al., 2002), by MKK9 during ethylene responses (Xu et al., 2008), by MKK3 in JA signal transduction (Takahashi et al., 2007), and by MKK1 during ABA-dependent cascade that leads to H₂O₂ production (Xing et al., 2008). Lastly, a single MAPK can phosphorylate multiple substrate proteins. For example, MPK6 can phosphorylate the rate-limiting ethylene biosynthesis enzyme, ASC6 (Liu and Zhang, 2004), the transcription factors SPEECHLESS (Lampard et al., 2008) and EIN3 (Yoo et al., 2008), and the ethylene response factor ERF104 (Bethke et al., 2009). Additionally, MPK3 can also phosphorylate the transcription factors SPEECHLESS (Lampard et al., 2008) and EIN3 (Yoo et al., 2008), and can interact with VIP1, a protein that facilitates the import of T-DNA protein complexes into the plant nucleus (Li et al., 2005). Due to the number of possible upstream and downstream components, MAPK signaling cascades form a
complex network that regulates a wide range of cellular processes. This leads to the fundamental question of how signaling specificity is established and controlled when only a few MAPKs mediate the response to a vast number of environmental cues.

MAP kinase signaling cascades are well characterized in animal and yeast systems, however, not as much is known about the organization of MAPK signaling modules in plants. There are multiple possibilities for the organization and establishment of specificity of MAP kinase pathways. To achieve signaling specificity, specific MAPK signaling components may be spatially regulated, which would restrict the interaction of the different signaling partners to a specific tissue or cellular compartment. Another mechanism, the temporal control of the different signaling events, could prevent or regulate specific interactions at distinct times. Another approach that may contribute to determining signaling specificity is the organization and recruitment of MAPK cascade signaling components by a scaffolding protein. In general, scaffolding proteins have multiple binding domains and are able to connect distinct proteins. Scaffold proteins can tether or co-recruit the specific components of a MAPK cascade to organize a signaling complex. Thus, scaffolds can act as a means to tightly regulate signaling pathways, signal intensity, duration, and/or cellular response (Brown and Sacks, 2009). In animal and yeast systems, MAPK scaffolding proteins have been shown to be involved in physically assembling the signaling components (Choi et al., 1994; Printen and Sprague, 1994), in control of signaling localization (Therrien et al., 1995; Garrenton et al., 2006; Winters et al., 2005), insulation of MAPK cascades from competing input signals (Patterson et al., 2010; Schwartz and Madhani, 2006), and are required components for proper signal transduction (Good et al., 2009).
In yeast, there are two MAPK pathways mediated by scaffold proteins, which are involved in determining the specificity and function of the signaling cascades. The scaffold protein Ste5 plays a vital role in binding the distinct MAPK components during the pheromone mating response. Ste5 is characteristic of most scaffolding proteins, in that it has distinct interaction domains that assemble specific kinases that are required for distinct cellular responses. During the perception of pheromones during the mating response, the Ste5 scaffold protein directs the phosphorylation of Fus3 (MAPK) by Ste7 (MAPKK), which results in a mating response (Malleshaiah, 2010). In contrast to this, under nitrogen starvation, Ste5 is inactive allowing Ste7 to phosphorylate Kss1 (MAPK), which leads to filamentation. A second pathway involves the scaffold Pbs2p, which is able to act as both a MAPKK and a scaffold protein in the coordination of an osmoregulatory MAPK pathway (Whitmarsh and Davis, 1998).

In mammals, the best-characterized scaffold involved in organizing MAPK signaling pathways is the protein Kinase Suppressor of Ras (KSR). KSR is required for the signaling by activated Ras and can bind all three kinase components of the MAPK cascade, Raf/MEK/ERK (Kortum and Lewis, 2004). KSR is able to interact with additional proteins such as 14-3-3 proteins, heat shock proteins 70 and 90, other kinases, and cell division control proteins (Brown and Sacks, 2009). Under non-stress conditions, MEK constitutively associates with KSR, whereas ERK only binds in response to a stimulus. KSR is able to modulate the specificity of ERK pathways, as well as facilitate the intensity and duration of ERK activation, which can have widespread effects on the ability of ERK to modulate an array of cellular processes (Kortum and Lewis, 2004).

In plants, MAPKs have been shown to interact with phosphatases, 14-3-3
proteins, and various substrate proteins (Rodriguez et al., 2010; Andreasson and Ellis, 2010; Oh et al., 2010). However, it has not yet been determined how and when these proteins interact with the MAPK signaling components. Plant MAPKs are crucial for initiating specific defense responses such as resistance to pathogens (Ekengren et al., 2003; Pedley and Martin, 2004) and defense against herbivores (Kandoth et al., 2007; Li et al., 2006; Wu et al., 2007). MAPKs must be able to integrate multiple incoming signals and subsequently activate specific substrate proteins inducing distinct output responses.

To date, there is little known regarding MAPK scaffold proteins in plants. In addition, there are no proteins with homology to animal or yeast scaffolding proteins present in plant genomes. Although the KSR and Ste5 both activate MAPK signaling pathways, they share minimal sequence similarity and most likely evolved independently. As with plants, the evolution of MAPK scaffolding proteins most likely occurred independently of animal and yeast systems.

In this study, we show that tomato MPK1/2 and MKK4, Arabidopsis MPK3 and MPK10, and tobacco SIPK interact with multi-protein complexes. We hypothesize that this contributes to the determination of signaling specificity. Based on the estimated size of the protein complex (~250-600 kDa), we postulate that additional proteins, such as scaffold proteins, MAPK phosphatases, MAPK substrates, MAPKKs, or MAPKKKs associate with these complexes. We found that only inactive (dephosphorylated) MAPKs associate with the multi-protein complex, unless treated with a phosphatase inhibitor, which allows association of phosphorylated MAPKs with the complex. Identification of plant MAPK scaffold proteins would help increase our understanding of how MAPK
signaling cascades are organized and regulated in response to the environment and external stimuli.

**Methods**

**Plant and Suspension-cultured cells**

Tomato (*S. lycopersicum* v. Rio Grande), tobacco (*N. tabacum*), *Nicotiana benthamiana*, and *Arabidopsis thaliana* were grown as previously described in Chapter 1. *Arabidopsis thaliana* seeds were sterilized briefly in ethanol (5 mins) followed by a 20% bleach solution containing 0.5% Tween-20 (5 mins), and washed 3 times in sterilized water. Seeds were vernalized in sterilize water for at least 48 hrs at 4°C, then germinated on ½ MS plates supplemented with 1% [w/v] sucrose for one week and transplanted to soil (Metro-Mix 360, Sun Gro, Agawam, MA) supplemented with perlite and vermiculite. *Arabidopsis* plants were grown similar to tomato, but at 25°C (16 hr daylight) and 22°C (8 hr night). AtMPK3 (at3g45640) T-DNA insertion mutant *mpk3-1* (SALK_151594), AtMPK6 (at2g43790) T-DNA insertion mutant *mpk6-2* (SALK_073907), and AtMPK10 (at3g59790) T-DNA insertion mutants *mpk10-1* (SALK_136149) and *mpk10-2* (SALK_039102) were ordered from ABRC (Alonso et al., 2003). *S. peruvianum* suspension-cultured cells (Felix, 1995; Yalamanchili, 2002) were cultivated in 125 mL Erlenmeyer flasks on an orbital shaker (200 rpm) at room temperature.

**Protein Extraction, Immunoblot Analysis and Immunoprecipitation**

Leaf tissue was extracted in a similar manner as described previously in Chapter 1. Briefly, frozen samples were homogenized with extraction buffer and centrifuged. Supernatant was used for determination of protein concentration. 30 µg to 50 µg of total
protein extracts were separated by SDS-PAGE, transferred to a PVDF membrane, and blocked in 5% [w/v] BSA. Blots were incubated with primary antibody overnight at 4°C. Primary antibodies used were anti-AtMPK6 (described in Chapter 1), anti-phospho-ERK (described in Chapter 1), anti-MKK4 generated against full-length tomato MAPKK4, anti-FLAG M2 (Sigma-Aldrich) used at a dilution of 1:7500, and anti-MPK3 (Sigma-Aldrich) at a dilution of 1:5000 in TBS-Tween 20.

For anti-FLAG immunoprecipitation (IP), SEC fractions were collected and samples were pooled from fractions corresponding to HMW or LMW fractions (2 mL total). The IP procedure followed the established protocol for anti-FLAG M2 affinity gel (Sigma-Aldrich) with minor variations. Briefly, 1% [v/v] Triton-X and 0.5% [v/v] nonidet P-40 were added to each pooled sample following SEC. Resin was prepared as described in the affinity gel manual with at least 40 µL of resin for each sample. Pooled samples (2 mL) were added to the prepared resin and placed on a roller shaker for two hours at 4°C. Samples were centrifuged and supernatant removed, then washed three times with TBS buffer (50 mM Tris HCl, with 150 mM NaCl, pH 7.4). Samples were eluted with 3X gel loading buffer (62.5 mM Tris HCl, pH 6.8, with 2% SDS, 10% [v/v] glycerol, and 0.002% [w/v] bromophenol blue, 50 mM 2-mercaptoethanol). Samples were boiled at 95°C for 5 minutes then subjected to SDS-PAGE and immunoblotting or coomassie staining.

**Treatments of Tomato Suspension Cells**

For treatments of samples used for immunoblotting, 1.5 mL of *S. peruvianum* suspension-cultured cells were added to each well of a 12-well tissue culture plate (BD Biosciences, San Jose, CA) and shaken on an orbital shaker at 150 rpm under ambient
room light and temperature. Cell suspensions were equilibrated for one hour prior to
treatment. Medium was removed from the samples using a Büchner funnel with
miracloth, and the cells were flash-frozen in liquid nitrogen. For size exclusion
chromatography, 125 mL Erlenmeyer flasks containing ~45 mL of cells were directly
treated and placed on an orbital shaker at 200 rpm in ambient room temperature and
light. 5 mL of cells were collected for each timepoint for further analysis. Cantharidin (Enzo
Life Sciences, Inc., Farmingdale, NY) was solved in DMSO and added to the cells for a
final concentration of 500 µM. Systemin (GenScript, Piscataway, NJ) was solved in
water and added to the cells for a final concentration of 10 nM.

Vector Construction and Transient Transformation of Nicotiana benthamiana

Full length tomato MPK1 was amplified from a Rio Grande cDNA library
and cloned into the pCR™8/GW/TOPO® vector (Life Technologies). TOPO vector was
transformed into competent DH5α E. coli cells and plasmid DNA was isolated from
positive transformants. Isolated plasmid recombined into the Gateway® vector,
pEarleyGate 202 (Earley et al., 2006), via an LR recombination reaction using LR
Clonase® II enzyme mix (Life Technologies) and transformed by electroporation into One
Shot® Mach1™-T1R chemically competent E. coli (Life Technologies). Positive
transformants were determined by restriction digest and confirmed by sequencing.
MPK1-FLAG plasmid was transformed into Agrobacterium tumefaciens strain GV3101.
One colony was selected and used to start an overnight in 3 mL of LB medium. Cells
used harvested by centrifugation, washed twice with 10mM MgCl₂ and resuspended in
infiltration medium [10mM MgCl₂, 10mM MES, and 150mM acetosyringone.(3’, 5’-
Dimethoxy-4’-hydroxyacetophenone, Acros Organics, New Jersey, USA)] to a final
OD$_{600}$ of 0.2 to 0.4. Infiltration medium was forced into abaxial side of *N. benthamiana* leaves and were sampled five days later.

**Size Exclusion Chromatography**

Total protein content was extracted from leaf tissue or suspension-cultured cells as previously described (Gusmaroli, 2004). Samples were homogenized with extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$, 2.5 mM EDTA, 25 mM β-glycerolphosphate, 10 mM Na$_3$VO$_4$, 5 mM NaF, 10 µM leupeptin, 1 mM PMSF, 1 mM DTT, 0.1% [v/v] Nonidet P-40, 10% [v/v] glycerol), centrifuged twice at 14,000 rpm for 10 mins at 4°C, and passed through 0.2 µm filters. Protein concentration was determined using Biorad Protein Assay Reagent (Biorad). Total protein extracts (500 µg) were injected onto a Superose 6 10/300 GLE column (GE Healthcare, Piscataway, NJ) previously equilibrated with equilibration buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$, 2.5 mM EDTA, 25 mM β-glycerolphosphate, 10 mM Na$_3$VO$_4$, 5 mM NaF). Samples were pumped through the column at a flow rate of 0.5 mL/min using an ÄKTA Explorer 100 (GE Healthcare). Fractions were collected in 0.5 mL increment to a total volume of 30 mL. Fractions including the high molecular weight complexes (~12 mL) and the fractions containing the monomeric proteins (~19 mL) were concentrated using StrataClean Resin (Aligent Technologies, La Jolla, CA), then washed briefly with sterile water and eluted by boiling with 3X SDS gel loading buffer. The entire sample, including resin, were separated by SDS-PAGE and analyzed via immunoblotting (described in Chapter 1).
**Trypsin Digest and Mass Spectroscopy**

Bands were excised from polyacrylamide gels using a clean scalpel. In-gel tryptic digestion kit (Thermo Scientific) was used for the fragmentation of immunoprecipitated proteins excised from the gel. The protocol was completed as described in the manual. Samples generated from digestion kit were purified and concentrated using PepClean™ C-18 spin columns (Thermo Scientific). 1 µL of both the sample and cinnamic acid MALDI matrix were spotted on a target plate. Peptides were analyzed on a Bruker Ultraflex MALDI TOF/TOF (Mass Spectroscopy Center, University of South Carolina). Results from MALDI-MS were used to search Mascot (Matrix Science).

**In-gel Kinase Assay**

Systemin-treated cell suspension extracts were analyzed by in-gel kinases assays using myelin basic protein (MBP) as an artificial MAPK substrate as previously described (Stratmann, 1997) with minor changes. Samples were homogenized with extraction buffer as described previously in Chapter 1. Protein concentration was determined using Biorad Protein Assay Reagent. 30µg of total protein extract was separated by gel electrophoresis on a 10% polyacrylamide gel containing 0.25 mg/mL myelin basic protein (Sigma-Aldrich). Proteins in the gel were denatured and renatured and the kinase reaction was performed as described previously (Usami, 1995). Briefly, following renaturation, gels are incubated with $^{32}$P-ATP for one hour then repeatedly washed to remove extraneous radiolabeled ATP. The gels were dried between cellophane sheets and radioactive signals were detected using a Storm phosphoimaging system (Amersham, Piscataway, NJ).
Results

Identification of a MAP kinase-containing multi-protein complex in tobacco, tomato, and Arabidopsis.

Initial evidence of a MAP kinase multi-protein complex was found in tomato plants and suspension-cultured cells. The complex was discovered using size exclusion chromatography (SEC) to initially separate proteins and protein complexes based on size, followed by immunoblot analysis of the elution fractions with an antibody that recognizes Arabidopsis MPK6, orthologs in tomato (MPK1 and MPK2) and tobacco (SIPK), and a closely related MPK6 paralog, Arabidopsis MPK10. We identified a high molecular weight (MW) complex (between ~400 and 600) in Arabidopsis, tobacco, and tomato. The complex contains Arabidopsis AtMPK10 (Figure 2.1A) and AtMPK3 (Figure 2.1B), tobacco SIPK (Figure 2.1C), tomato MPK1/2 (Figure 2.2A upper panel) and tomato MKK4 (Figure 2.1D). MAPKs also eluted in lower molecular fractions (~150 to <50 kDa), which represent the generally larger pool of monomeric MAPK proteins.

The biphasic elution profile is consistent with all wild type plants and suspension cells analyzed. To confirm that the high MW proteins correspond to MAPKs and not a cross-reacting protein, leaf tissue from Arabidopsis T-DNA insertion mutants were analyzed. Wild type Columbia-0 plants showed both high and low MW bands when probed with anti-AtMPK6 and anti-AtMPK3 (Figures 2.1A and 2.1B, upper panels). When probed for MPK6, we found that only the lower MW bands correspond to MPK6 (Figure 2.1A, second panel). The proteins eluted in high MW fractions were determined to be MPK10 (Figure 2.1A, third and fourth panels). The MPK10 amino acid sequence shares 8 sequential residues with the peptide sequence used to generate Anti-AtMPK6 antibodies (data not shown). The high MW bands remain in mpk6-2 (Bush and Krysan,
and are missing in two MPK10 T-DNA insertion mutants, *mpk10-1* and *mpk10-2*. The opposite is observed for the low MW bands when probed with anti-AtMPK6, which indicates MPK10 associates with a complex, but not MPK6. MPK3 elutes in both high and low MW fractions, which are not present in *mpk3-1* plants (Wang et al., 2007; Figure 2.1B, lower panel). A MAPK-containing complex was also identified in tobacco, which elutes at a similar size and has a similar elution profile with both monomeric and complex-associated SIPK accumulation. In tomato, MPK1/2 was observed to associate with a protein complex (Figure 2.2A, upper panel). The proteins in high and low MW fractions were confirmed as MPK1/2 by examining tomato MPK1/2-VIGS silenced plants (Hind and Stratmann, unpublished data). It is surprising that MPK6 orthologs in tomato and tobacco associate with the high MW complex, whereas *Arabidopsis* MPK6 does not. Instead, a close MPK6 paralog, MPK 10, and MPK3, which also share a high level of homology with MPK6 (74% similar to MPK3, 71% similar to MPK10 at the amino acid level), clearly associate with a high MW complex. Additionally, the tomato MAPKK MKK4 was identified in both high and low MW fractions in suspension-cultured cells (Figure 2.1D). Together, these results demonstrate the existence of a high MW MAPK multi-protein complex (or complexes) in plants that associate with both MAPKs and MAPKKs.

**Active MAPKs do not associate with MAPK-containing complexes**

In previous studies in animals and yeast, MAPK scaffolding proteins have been shown to play a role in organizing and orchestrating MAPK signal transduction events. To evaluate if the MAPK complexes identified in tomato, tobacco, and *Arabidopsis* are involved in signal transduction, we activated MAPKs using the wound signaling peptide
Figure 2.1: Identification of a MAP kinase-containing multi-protein complex in *Arabidopsis*, *tobacco*, and *tomato*. Total protein (300 to 750 µg) was extracted from leaf tissue and separated by size exclusion chromatography. Fractions (0.5 mL) were collected and concentrated. Samples were separated via SDS-PAGE, immunoblotted, and probed with the antibodies specified. 

A) Immunoblots of Columbia-0, *mpk6*-2, *mpk10*-1, and *mpk10*-2 fractions (13 to 18.5 mL) were probed with anti-AtMPK6 (α-MPK6). The input in lane 1 represents 30 µg of total protein extracted from Col-0 (all panels), compared to total proteins from *mpk6*-2 (second panel from top), and *mpk10*-1 (third panel from top) in lane 2. 

B) Immunoblots of *Arabidopsis* Col-0 and *mpk3*-1 total protein separated by SEC, immunoblotted, and probed with anti-AtMPK3. Inputs represent Col-
hormone, systemin, and analyzed the protein complexes. Samples were evaluated for the association of phosphorylated MAPKs with the high MW complex using SEC and immunoblotting. Tomato suspension-cultured cells were treated with systemin, which is known to cause rapid activation of MPK1/2 (Kandoth et al., 2007). Samples were fractionated by SEC, subjected to immunoblotting and probed with anti-pERK, which specifically detects MAPKs with dual-phosphorylation of the T-X-Y motif in the activation domain. Only dually phosphorylated MAPKs exhibit enzyme activity. Therefore, anti-pERK can be used for the detection of active MAPKs, while anti-AtMPK6 recognizes active and inactive MPK6 and orthologs. Using the anti-AtMPK6 antibody, we show that tomato MAPK1/2 elutes in high and low MW fractions in tomato suspension cells (Figures 2.2A and 2.2B, upper panels). Using the anti-pERK antibody, there is no accumulation of active MPK1/2 in either high or low MW fractions prior to systemin treatment (Figure 2.2A, middle panel). After 10 minutes of systemin treatment, there is rapid accumulation of active MPK1/2 in low MW fractions, but there is no accumulation in high MW fractions (Figure 2.2B, middle panel). These immunoblot results were confirmed using an in-gel kinase assay (IGKA) as an independent enzymatic approach to examine the relationship of phosphorylated MAPKs and protein complexes. IGKA uses myelin basic protein (MBP) as an artificial MAPK substrate, which can be
phosphorylated by MAPKs using radiolabeled ATP. The IGKA confirms the immunoblot experiments that active MPK1/2 is only present in low MW fractions. These results indicate that inactive but not active MAPKs associate with the protein complex, which suggest a potential role of the complexes in MAPK deactivation.

Figure 2.2: Active MPK1/2 does not associate with a multi-protein complex in tomato. *Solanum peruvianum* suspension-cultured cells were treated with 10 nM systemin for 10 mins (B) or left untreated (A). Total protein was extracted from the collected cells then separated by SEC, immunoblotted, and probed by anti-AtMPK6 (top panel: A, B) or anti-pERK (middle panel: A, B). or used for in-gel kinase assays following SEC separation (lower panel: A, B). Inputs represent 30 µg of total protein extracted from cells collected at the times indicated after systemin treatment. Immunoblot data courtesy of Hind and Pulliam (unpublished data).
Phosphatase inhibition alters MAPK-containing complexes

To further examine the role of MAPK-containing complexes for MAPK activation, tomato suspension-cultured cells were treated with the potent serine/threonine phosphatase inhibitor, cantharidin. We tested the effect of cantharidin on tomato cells over a six-hour period and observed an initial transient activation of MPK1/2 at 10 minutes, followed by a gradual increased accumulation of MPK1/2 (48 kDa), MPK4 (44 kDa) and most likely MPK3 (middle band; Figure 2.3A). MAPK activation by phosphatase inhibitors is a known phenomenon and thought to be caused by a lack of MAPK dephosphorylation, which is important to maintain a low background activity of MAPKs (Zhao et al., 1996). Samples of cells treated for six hours with cantharidin were subjected to SEC and immunoblot analysis for the association of active MAPKs in the complex. In these samples, MPK1/2 accumulated in low MW fractions as observed previously in Figure 2.2. In contrast, in cantharidin-treated cells, active MPK1/2 was present in high MW fractions (Figure 2.3B). To confirm these results, we analyzed cantharidin-treated suspension cells at 10, 120, and 180 minutes. We observed elution of active MPK1/2 in high MW fractions in samples treated for 120 and 180 minutes, but not at 10 minutes (Figure 2.3C). These results demonstrate that active MAPKs can associate with the protein complex after extended treatment with a phosphatase inhibitor.

Identification of MAPK-containing complex proteins

The evidence found here suggests that MAPKs associate with a multi-protein complex, therefore, it is important to identify the components of the complex in order to better understand how MAPK signaling is regulated in response to a stimulus. Since MAPKs are the only known proteins that part of the complex, we generated a vector that
Figure 2.3: Active MPK1/2 associates with a multi-protein complex after cantharidin treatment. S. peruvianum cells were treated with 500 µM of the phosphatase inhibitor cantharidin and sampled at the times indicated. A) Total protein was extracted from suspension-cultured cells untreated (0 min) or treated with cantharidin, immunoblotted, and probed with anti-pERK antibody. Arrows indicate MPK1/2 (48 kDa) and MPK4 (44 kDa). B) Tomato cells collected 360 mins after treatment with cantharidin were extracted, separated by SEC, immunoblotted, and probed with anti-pERK. Immunoblot includes fractions 13 to 18.5 mL eluted from the column. Input represents 30 µg of total protein. C) Tomato cells treated with cantharidin for the duration indicated. Each timepoint represent the same blot probed with anti-pERK (upper panel), then stripped and re-probed with anti-AtMPK6 (lower panel). Blots include fractions 13 to 15.5 mL.
expresses tomato MPK1 N-terminally fused to FLAG under control of a 35S promoter. The general approach used to identify complex-associated proteins is diagramed (Figure 2.4). *Agrobacterium tumefaciens* containing tomato MPK1-FLAG was syringe-infiltrated into the leaves of *N. benthamiana*. Tomato MPK1 and NbMPK1 are 95% similar at the amino acid level. Plants were sampled 5 days after infiltration and expression and accumulation of MPK1-FLAG was confirmed by IB and probed with anti-FLAG (Figure 2.5B, inputs). Total protein extracted from either non-infiltrated *N. benthamiana* (wild type, WT) or MPK1-FLAG-infiltrated plants was separated by SEC and analyzed by IB and probed with anti-FLAG (Figure 2.5A). High molecular weight (HMW) and low MW (LMW) fractions were collected from 13 to 14.5 mL eluted from the column and 15.5 to 17 mL, respectively. Anti-FLAG antibody detects a cross-reacting protein around 48 kDa, which is approximately the same size as MPK1-FLAG. HMW and LMW fractions were pooled and immunoprecipitated using anti-FLAG M2 affinity gel. IP Samples were either analyzed by IB (Figure 2.5B, right panel) or subjected to SDS-PAGE and stained with coomassie (Figure 2.5C). IP samples demonstrated patterns of MPK1-FLAG accumulation similar to Figure 2.5A, where LMW fractions had a greater abundance of MPK1-FLAG compared to HMW fractions that had less MPK1-FLAG (Figure 2.5B).

Areas of the gel where bands were present in MPK1-FLAG IP lanes and not present in WT lanes were excised and digested by trypsin. Peptide fragments were further purified and concentrated on C-18 columns, then analyzed by MALDI-TOF/TOF mass spectroscopy. The digestion of the entire MPK1-FLAG HMW lane (Figure 2.5C, black box, except the dark blue band, which was excised and analyzed separately) identified a potential candidate protein, a MAPKKK. This is a protein that may be involved with the
complex, although, the data indicates that this result could also be a random match (low sequence coverage). We were able to demonstrate a method to isolate MAPK-containing protein complexes using epitope tagged MPK1 and identify a candidate protein, albeit, one that may not be a true candidate.

Figure 2.4: Flow diagram for the identification of MAPK complex proteins.
Diagram for the steps to identify components of the MAPK complex. N-terminal tagged tomato MPK1 was transiently transformed into *N. benthamiana*. Total protein extracted and separated by SEC. Samples confirmed by immunoblot for accumulation of MPK1-FLAG in HMW fractions. Next, samples were immunoprecipitated using anti-FLAG M2 affinity gel. Samples were pooled for fractions that represent HMW or LMW fractions. Pull-down of MPK1-FLAG confirmed by immunoblot. Next, samples were subjected to SDS-PAGE and stained with coomassie. Bands or lanes were excised from the gel and subjected to in-gel tryptic digest followed by analysis by mass spectroscopy. Results from MS analysis used for database search (MASCOT) to identify candidate proteins.
Figure 2.5: Mass spectroscopic analysis of a MAPK complex candidate protein.

A) Total protein from wild type or MPK1-FLAG transiently transformed *N. benthamiana* was extracted five days after infiltration of *Agrobacterium*-containing MPK1-FLAG. 1 mg of total protein was separated by SEC and analyzed via immunoblot (IB) and probed with anti-FLAG. IB shows fractions 13 to 18.5 mL with high MW (HMW) and low MW (LMW) fractions indicated.

B) Total protein was extracted and separated by SEC and fractions corresponding to either HMW or LMW were pooled and immunoprecipitated using anti-FLAG M2 affinity gel. Samples were eluted in loading dye and analyzed via immunoblots and probed with anti-FLAG. Inputs are 50 µg total protein extracted from WT MPK1-FLAG.

D. Predicted:
**MAP kinase kinase kinase 10-like**
Nominal Mass (M): 66343
Calculated pI: 5.98
Sequence coverage: 14%

```
1 MAAALECWSS RTTAAATST DDDTVEQVLM RTHHRSEGTT TTSTPNNN
51 KDPSAIPVH K Kelagqktrnv seeiasfrns L NLSDPSRSSK ADASSRKLAW
101 GSVRNLQQL YPGSGLPEKL MSNIRKHYS LPLSAYAQAEF D MKVDVHLIK
151 LMEQASESDD PAILQEEDY YEVRQIALRL TFACNSPISW PAMSGLDSS
201 SICRKNMQIF EKKGRLGVV LLVQSGHDK LRVRVESAL KPAMKRPITG
251 AVKLPPGLGC CQEEENKSGD LVEIEEIESD CYRGKEPENS GQTIQLQVPL
301 PSSSFVVSVD EMQTKSNGD EIEKWLNSD SVEFVEQIGP NFGKGVYLGK
351 RKIIEKLGC DKNSYEELI HKDLEELMHC GHRNLQFCGC ICVDNHHGLC
401 VVTKFMEEGGS VHDLMKKNKQ LQKD KDIVIK VDVAIEIKFM NDHVAYRDAL
451 NTPRILLDRH GNACLGDGI VTAACKVGEA MEYETDGYRW LAPEIIAGDP
501 ENVETWMSN VYSFHVIWE MVTGETAYSS FPVPQAVGIG AACGLRPEIP
551 KDCQQTLYI MTKCWNNNPS KRPHFSEILA ILLPNNNRR
```
WT and MPK1-FLAG and analyzed by immunoblot. C) Total protein was extracted and separated by SEC and fractions were pooled from HWM (H) or LMW (L) fractions and immunoprecipitated using anti-FLAG M2 affinity gel. Samples were subjected to SDS-PAGE and stained with coomassie blue (CBB). Black rectangle indicates lane used for MS. Inputs represent 50 µg of total protein subjected to SDS-PAGE. D) Lane containing HMW fractions from MPK1-FLAG were excised from the gel was digested using trypsin. Peptide fragments were analyzed by MS. Database search (MASCOT) identified a possible candidate protein.

Discussion

The study of MAPK signaling in plants has been mostly limited to the investigation of small pieces of a larger puzzle, however, it is becoming more obvious that MAPK signaling pathways are intricate global networks involved in many aspects of plant biology. Previous studies have demonstrated that some MAPKs are involved in multiple signal transductions pathways. Based on the number of known and predicted MAPKs (20 in Arabidopsis), MAPKKs (10), and MAPKKKs (~80), there are many possible combinations of these three different signaling components, and these combinations are known to mediate specific output responses. In addition, a single MAPK can be activated by multiple MAPKKs and itself can phosphorylate multiple substrate proteins (MAP Kinase Group, 2002; Andreasson and Ellis, 2010). Due to this promiscuity of MAPKs, there must be tight regulation and organization of MAPK signaling modules to ensure proper transduction of a perceived extracellular stimulus. The mechanism of MAPK signaling may be either a stepwise interaction between MAPKKK, MAPKK, and MAPK, or all the signaling partners organized into a single signaling module mediated by a scaffolding protein, which would bring together each of the proteins into one complex (Whitmarsh and Davis, 1998).
Scaffold proteins have multiple distinct protein binding domains, which can associate with multiple binding partners to bring them into close proximity to one another. This can result in the insulation of MAPKs from aberrant signaling. Organization of MAPKs via scaffolding proteins may also control the specific subcellular localization providing access to co-localized MAPK substrate proteins, and it may affect timing and duration of MAPK signaling. These factors all contribute to signaling fidelity (Pouysségur et al., 2002). Studies in yeast and animals have discovered MAPK scaffolding proteins that are critical for proper cellular responses, however, there is very little known about MAPK scaffolds or complexes in plants.

In this study, we identified a MAPK-containing complex that is present in tomato, tobacco, and Arabidopsis. The complex elutes in fractions that are approximately 300 to 600 kDa in size, which indicates the complex consists of multiple proteins. We show that both MAPKs and MAPKKs are able to associate with a larger protein complex. Assuming MWs of generally larger than 40 kDa for MAPKs and MAPKKs and sometimes substantially larger for MAPKKKs, the protein complex we identified is likely to contain additional proteins such as known interactors MAPK modules (protein phosphatases, 14-3-3 proteins, MAPK substrates) and possibly proteins with scaffolding function. A previous study in Medicago identified a novel MAPKKK OMTK1, which is able to directly interact with and activate a MAPK, MMK3, in response to H$_2$O$_2$ (Nakagami et al., 2004). They also suggest that OMTK1 acts as a scaffold for MMK3 and an unknown MAPKK. Additionally, MPK4 is part of a complex with a nuclear substrate, MAP KINASE SUBSTRATE 1 (MKS1) and the transcription factor WRKY33. Pathogen infection or flg22 treatment results in the release of WRKY33 from a complex with
MPK4, MKS1, or both, and then binds to and activates the transcription of the camalexin biosynthesis enzyme PAD3 (Andreasson et al., 2005).

We show that tomato MPK1/2 and its tobacco homolog, SIPK, associate with a protein complex, however, the Arabidopsis homolog, MPK6, only elutes in low MW fractions and does not appear to be part of a larger protein complex. Our data do not show that MKK4 and MAPKs share the same protein complex, but it is consistent with a complex that organizes at least two members of the MAPK cascade. In Arabidopsis, we show that MPK3 and MPK10 associate with the complex. MPK3 is well characterized for its role in plant responses, however, much less is known about MPK10, which is part of subgroup A of Arabidopsis MAPKs that also includes MPK3 and MPK6. AtMPK10 has low basal expression and protein levels, but is transiently expressed in seedlings and at sites of auxin concentration maxima (Stanko et al., 2014). There is some evidence to suggest MPK10 plays a role in plant defenses, where overexpression of MPK10 triggered cell death (Popescu et al., 2009). Association of Arabidopsis MAPKs with a protein complex were confirmed using T-DNA insertional mutants, which showed loss of both high and low MW MPK3 protein in mpk3-l and loss of high MW MPK10 protein in mpk10-l and mpk10-2. The loss of low MW MPK10 could not be clearly demonstrated in the mutants, which may be due to the use of anti-AtMPK6, which would recognizes both the low MW MPK10 and MPK6. This makes observing the loss of MPK10 from low MW fractions difficult in mpk10-l and mpk10-2.

Examining the signaling and activation dynamics of MAPKs and how they associate with a protein complex revealed that only inactive MPK1/2 associates with the complex, whereas active MPK1/2 only associates with the complex after treatment with a
phosphatase inhibitor. This implies the protein complex may play a role in the regulation of MAPK activation or deactivation. There are a few possibilities to explain these results. First, MAPK phosphatases may associate with the complex, which would function to dephosphorylate the MAPKs as they associate with the complex. This would serve to recycle MAPKs following activation in order to keep a pool of inactive kinases readily available for quick phosphorylation and activation in response to a stimulus. Second, the complex may constitutively associate with inactive MAPKs and only during stress responses interact with MAPKKKs or MAPKKs to induce the signaling cascade. Thus, activation of MAPKs could lead to their dissociation from the complex. If phosphatases are inhibited, e.g. by cantharidin, it may alter the basal dephosphorylation of MAPKs resulting in the increased accumulation of active MAPKs, which could tip the equilibrium of complex-associated MAPKs from unphosphorylated to phosphorylated MAPKs. We observed the accumulation of active MAPKs in response to cantharidin treatment alone (Figure 2.3A). It is noteworthy that in most plants we investigated, the majority of MAPK proteins seem to be present in the monomeric form. This could be either an artifact due to disruption of the complex during sample processing, or it may indicate that complex-associated MAPKs represent a subpool with specialized functions.

An important next step in this study is the identification of MAPK complex protein constituents. The identification of the protein components of the complex is difficult since the only known members of the complexes are MAPKs or MAPKKs. To identify the proteins involved in forming the complex, we have tried multiple approaches such as immunoprecipitation using antibodies against the various MAPKs, epitope tagged purification of the complex, and tandem affinity purification of the complex. N.
*benthamiana* transiently transformed with MPK1-FLAG showed elution of MPK1-FLAG in HMW fractions, indicating that is fusion protein is able to associate with the protein complex. The accumulation of MPK1-FLAG in LMW fractions is likely due to the strong expression of MPK1-FLAG and only a limited number of scaffolding proteins that can associate with MPK1-FLAG proteins. We identified the candidate protein, MAP kinase kinase kinase 10-like, through the pull-down of MPK1-FLAG and associated proteins followed by MS analysis. Although the sequence coverage is low and may not be a true result, this is a promising approach to identify the components of the complex. Using a similar approach but without the SEC step, we were able to identify MPK1-FLAG by MS, which serves as a proof of concept. We have tried other epitope tags, such as HA and c-myc, however these epitope-tagged MAPKs did not associate with the complex, which is most likely due to interference from the tag with the binding site of the scaffold. Further studies are needed to determine what proteins are part of the MAPK complexes.

Although there is a prior study that identified a putative MAPK scaffold protein, our study is the first to identify a protein complex that is found in multiple plants, associates with multiple MAPKs and a MAPKK, and plays a role in the dephosphorylation of MAPKs. This work identifies an important aspect of MAPK signaling that has not been previously identified in plants. This work further increases our understanding of how MAPK cascades are organized and regulated in plants and is an important step in the further identification of MAPK signaling components.
Chapter 3: Characterization of *Arabidopsis* MAPK phosphatases during defense responses.
Abstract

MAP kinase signaling cascades are one of the main phosphorylation signal transduction pathways in eukaryotes, which are involved in the regulation of many cellular functions. Tight regulation of MAPK signaling amplitude and duration are required for the proper outcome of MAPK signal transduction pathways. MAPK activity is regulated by MAPK kinases that activate MAPKs via phosphorylation and by MAPK phosphatases that inactivate MAPKs through dephosphorylation. Reversible phosphorylation determines the duration and amplitude of MAPK activity, which affects the specificity of the output response. There are 11 known plant MAPK phosphatases in Arabidopsis that can interact with MAPKs, however, there is limited evidence of how MAPKs are regulated during defense responses to herbivores and pathogens. By testing Arabidopsis T-DNA insertional knockout mutants for 7 MAPK phosphatases, we report novel roles of MAPK phosphatases in the regulation of responses to the bacterial elicitor, flg22 and pathogenic bacteria (Pseudomonas syringae), and to mechanical wounding and herbivory (Bradysia sp.). In most of the MAPK phosphatase knockout mutants, MAPK activation is enhanced and prolonged in response to wounding and elicitation by flg22. Wound-induced JA accumulation and SA levels are unaltered in these mutants. Preliminary data indicate that some MAPK phosphatase mutants are more susceptible to herbivory by Bradysia and more resistant against infection by P. syringae. These data present the basis for further research aimed at a detailed physiological characterization of MAPK phosphatases during biotic stress responses.
**Introduction**

In plants, MAPK activation by phosphorylation is well understood, however, little is known about MAPK inactivation by dephosphorylation. An appropriate cellular outcome of MAPK signaling pathways is highly dependent on strict regulatory mechanisms acting on MAPKs (McClean et al., 2007). MAPKs require dual-phosphorylation of both the threonine and tyrosine residues in the T-X-Y phosphorylation motif for activation (X is either glutamic acid or aspartic acid), however, the dephosphorylation of one or both phosphorylated amino acid residues is sufficient to inactivate MAPKs (Camps et al., 2000). Protein phosphatases dephosphorylate and inactivate MAPKs, which counterbalances the activation of MAPKs by MAPKKs. There are three classes of protein phosphatases that have been shown to play a role in the dephosphorylation and inactivation of MAP kinases: tyrosine specific phosphatases (PTPs), serine/threonine phosphatases, and dual-specificity phosphatases (DSPs), which can dephosphorylate both phosphoserine/phosphothreonine and phosphotyrosine residues (Gupta, et al., 1998). A subgroup of dual specificity phosphatases has been shown to be specific MAPK phosphatases (Camps et al., 2000). There is limited research on phosphatases that are involved in regulating MAPK activity and it is not well understood how dephosphorylation by phosphatases is able to influence the physiological result of MAPK cascades, especially in response to herbivory and pathogenic bacteria.

Currently, there are 11 phosphatases that are known to interact with MAPKs, of which five are DSPs, three are serine/threonine phosphatases, and one is a PTP. There are five PP2C-type serine/threonine phosphatases, ABI1, AP2C1 to AP2C4 (AP2C3 is also known as PP2C5). ABI1, or ABSCISIC ACID INSENSITIVE1, can directly interact with
MPK6 \textit{in vitro} and in yeast, and is implicated in abscisic acid-dependent signaling (Leung et al., 2006). The AP2C family of phosphatases is able to inactivate MAPKs by dephosphorylation of the threonine residue in the T-X-Y motif. All four AP2C phosphatases can inactivate MPK6 in \textit{Arabidopsis} and can interact with MPK6, MPK3, and MPK4. The \textit{ap2c1} mutant demonstrates elevated wound-induced JA accumulation and resistance against phytophagous mites, while overexpression resulted in compromised innate immunity against necrotrophic pathogens (Schweighofer et al., 2007). PP2C5 (AP2C3) is involved in the regulation of seed germination, stomatal aperture, and abscisic acid-inducible gene expression (Brock et al., 2010). AP2C1 and AP2C3 are the best studied AP2C phosphatases, whereas, there is little known about AP2C2 and AP2C4.

There is only one PTP-type phosphatase, PTP1, which is able to dephosphorylate MPK 4 and MPK6. PTP1 expression is altered in response to various environmental stimuli (Xu et al., 1998). In addition, hydrogen peroxide activates MPK6 and inactivates PTP1 (Gupta and Luan, 2003). There are 5 DSP-type phosphatases that are either predicted or known to be involved in MAPK dephosphorylation. IBR5 is involved with abscisic acid and auxin signaling (Monroe-Augustus et al., 2003), and has been shown to interact with \textit{Arabidopsis} MPK12 (Lee et al., 2009). PHS1 is associated with microtubule organization (Naoi and Hashimoto, 2004) and can interact with MPK18 (Walia et al., 2009). MKP1 is involved in the negative regulation of MPK6-mediated PAMP responses, resistance against bacteria, and is involved in genotoxic and salt stress tolerance (Anderson et al., 2011; Ulm et al., 2001 and 2002). MKP2 can dephosphorylate MPK3 and MPK6 \textit{in vitro} and is a positive regulator of oxidative stress tolerance (Lee and Ellis,
MKP1 and PTP1 also function as repressors of salicylic acid synthesis (Bartels et al., 2009). DsPTP1 (not to be mistaken for PTP1) can dephosphorylate *Arabidopsis* MPK4, however, the physiological and *in vivo* role of DsPTP1 is currently not known. Many of these phosphatases have been described genetically, however, the specific *in vivo* functions are not well understood.

In animals, MAPKs p44 and p42 target the MAP kinase phosphatase MKP-1, which phosphorylate MKP-1 on two serine residues. This phosphorylation stabilizes MKP-1 and reduces its degradation by the ubiquitin-directed proteasome system. Therefore, active p44 and p42 stabilize and reduce the degradation of the phosphatase responsible for their dephosphorylation, which is important for limiting the activation of p44/p42 (Brondello, et al., 1999). This demonstrates that MAPKs and MAPK phosphatases are part of a complex mechanism of feedback regulation of MAPK activity. MAPK phosphatases associate with MAPKs via MAPK binding domains, which causes a conformational change to the MAPK phosphatase. This results in a catalytically active conformation where the phosphorylated residues of active MAPKs are dephosphorylated (Stewart et al., 1999; Theodosiou and Ashworth, 2002).

This study aims to evaluate the function of *Arabidopsis* MAPK phosphatases in response to herbivory and pathogens. AP2C1 and MKP1 have been previously shown to be involved in the regulation of MPK6 in response to perception of PAMPs and bacteria (Schweighofer et al., 2007; Anderson et al., 2011). Additionally, MKP1 can negatively regulate the wound response and induced resistance to necrotrophic pathogens and herbivores (Oka et al., 2013). Here, we systematically examine MAPK phosphatases in the regulation of MAPKs during biotic stress responses.
Methods

MPK Phosphatase T-DNA Insertion Mutants

MPK phosphatase T-DNA SALK lines were obtained from ABRC (Ohio State University, Columbus, OH). Lines were tested for mutant allele homozygosity via DNA extraction and subsequent amplification by PCR. DNA was extracted from one rosette leaf of 10-14 day old seedlings by grinding in a 1.5 mL microcentrifuge tube with extraction buffer (200 mM Tris (pH=7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% [w/v] SDS). DNA was precipitated with isopropanol, washed with 70% ethanol, air-dried, and resuspended with sterile water. DNA was used for PCR analysis using primers specific for areas both upstream and downstream of the predicted T-DNA insertion and a primer for the left border of the T-DNA, LBb1.3 (LBb1.3 primer – ATTTTGCCGATTTTCGG AAC; MAPK T-DNA phosphatase mutant primer sequences are listed in Table 3.1).

Mechanical Wounding and Elicitor Treatments

For wounding experiments, plants were mechanically wounded using serrated hemostats. To ensure equal wounding for all samples, plants were wounded with only the tip of the hemostat (~1 cm) on both sides and parallel to the midvein, which was repeated rapidly on 6 to 8 rosette leaves from multiple plants. Timepoints were collected by excision and flash frozen in liquid nitrogen. Total protein was extracted and subjected to SDS-PAGE and immunoblotting using anti-phosphoERK antibody (see Chapter 1).

For elicitor treatments, leaf disks were excised from plants using a 4 mm cork borer and placed into the wells of a 12 well tissue culture plate (BD Biosciences). For each sample, 10 to 15 leaf disks were placed abaxial side downward in a well filled with water. Since excision of the leaf disk induces MAPK activation, leaf disks were left
overnight for at least 16 hours before treatment. Leaf disks were placed on an orbital shaker at 100 rpm for one hour prior to elicitor treatment to allow for adjustment. To treat the leaf disks, the water was removed from each well and replaced with 1 mL of a 25 nM Flg22 treatment solution. Samples were collected at the indicated timepoints and analyzed as previously described by protein extraction, SDS-PAGE, and immunoblotting using anti-phosphoERK antibody.

**Salicylic Acid and Jasmonic Acid Analysis**

Measurement of SA and JA levels was performed as described in Chapter 1. *Arabidopsis* plants between 4 and 6 weeks were used for analysis. Plants were grown as described in Chapter 2, but were grown under a short-day regime with 8 hrs of daylight at 25°C and 16 hrs of darkness at 22°C, which causes enlarged rosette leaves to facilitate obtaining sufficient tissue for analysis (~300 mg/sample). For wounded samples, each leaf was wounded twice with a hemostat parallel to the midvein, then excised and flash frozen after one hour.
Table 3.1: *Arabidopsis thaliana* MAPK phosphatase T-DNA insertion null mutants and genotyping primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>SALK Seed  Stock</th>
<th>Genotyping LP Primer</th>
<th>Genotyping RP Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI1</td>
<td>AT4G26080</td>
<td>SALK 072009</td>
<td>5’-TGAATATAGGAAGTCTGAAAGCAAGT3’ 5’-CGAAACAGCATCTCCATCTC-3’</td>
<td></td>
</tr>
<tr>
<td>AP2C1</td>
<td>AT2G30020</td>
<td>CS879551</td>
<td>5’-CGTTTGCATGAAACAGATTG-3’ 5’-CTCTGGTAAACGAAGGGAAACC-3’</td>
<td></td>
</tr>
<tr>
<td>AtPTP1</td>
<td>AT1G71860</td>
<td>SALK 118658</td>
<td>5’-AGAAAGGTCAATTCTCCGC-3’ 5’-TAGTCCCCCGCATTAACAGTC-3’</td>
<td></td>
</tr>
<tr>
<td>DsPTP1</td>
<td>AT3G23610</td>
<td>SALK 092811</td>
<td>5’-TCCCTCTTCTTAATTGAACAGG-3’ 5’-AAACAATGACAAGCATGAAC-3’</td>
<td></td>
</tr>
<tr>
<td>IBR5</td>
<td>AT2G04550</td>
<td>SALK 032185</td>
<td>5’-GAGATTTCTGGCAATAGGAACCT-3’ 5’-TCTCCTGGTCATTTGGTTG-3’</td>
<td></td>
</tr>
<tr>
<td>MKP1</td>
<td>AT3G55270</td>
<td>SALK 003028</td>
<td>5’-ATAGCACCACGGATGTTCAGG-3’ 5’-GCATTGTTTTGATATTGTATG-3’</td>
<td></td>
</tr>
<tr>
<td>PHS1</td>
<td>AT5G23720</td>
<td>SALK 062457</td>
<td>5’-TATGGCAACAGGTGTAAAATC-3’ 5’-AGTAATCATCATCCGGCACAGGC-3’</td>
<td></td>
</tr>
<tr>
<td>PP2C5</td>
<td>AT2G40180</td>
<td>SALK 015191</td>
<td>5’-CATGCATTTCGATAGGTGATC-3’ 5’-TGTAACCTCCGTCGTCATT-3’</td>
<td></td>
</tr>
</tbody>
</table>

Results

Role of MAPK phosphatases during defense responses to wounding

In order to examine the function of MAPK phosphatases in response to pathogens and herbivores, we obtained *Arabidopsis* T-DNA insertion null mutants for all available phosphatases (ABRC). We were able to confirm or establish 7 homozygous mutant lines: *dsptp1, mkp1, phs1, abi1, ibr5, pp2c5*, and *ap2c1*. T-DNA insertion mutant lines were not available for MKP2, AP2C2, or AP2C4, and we were unable to establish a homozygous line for PTP1. Mechanical wounding with a hemostat is sufficient to activate MAPKs, therefore, we used this approach to determine if the loss of individual MAPK phosphatases alters MAPK signaling dynamics. In response to wounding of *Arabidopsis* rosette leaves with a hemostat, MPK6 and MPK3 are activated within 15 minutes and
activity returns to basal levels between 30 to 60 minutes (Figure 3.1). The phosphatase mutant lines demonstrated varying responses to the mechanical wounding. We observed prolonged phosphorylation of both MPK3 and MPK6 up to 60 minutes following wounding of ap2c1 and dsptp1 mutants compared to Col-0 plants. Weaker prolonged activation was observed in mpk1, ibr5, abi1, phs1, and pp2c5 mutants at 30 minutes. All mutants except ibr5, abi1, and pp2c5 showed prolonged MPK6 activation at 60 minutes. Constitutive activation of MPK6 and MPK3 in unwounded ap2c1, and slightly in ibr5, abi1, and dsptp1 plants, was observed. Active MPK4 slightly accumulates in ap2c1, dsptp1, and pp2c5 at 30 and 60 minutes. In a separate wounding experiment, ibr5, mkp1, and dsptp1 showed increased phosphorylation of MPK6 and MPK3 after 15 min of wounding compared to wild type plants, and prolonged phosphorylation at 30 min compared to wild type plants (data not shown). Together, this indicates that multiple MAPK phosphatases, especially AP2C1 and DsPTP1, may be involved in dephosphorylating MPK6, MPK3, and to some extent, MPK4 in response to wounding.

One of the main defense responses triggered during herbivory is the biosynthesis and accumulation of the plant hormone, jasmonic acid and its derivatives. Since MAPK phosphorylation in response to wounding was more prolonged in most of the MAPK phosphatase mutant lines compared to the wild type, we measured the accumulation of JA after mechanical wounding, which depends on MAPK signaling (Kandoth et al., 2007). JA levels in unwounded plants were similar to Col-0 (145.8 ± 48.9 pmol/g fresh weight) in all lines tested and increased upon wounding within one hour (6637.8 ± 2571.5 pmol/g FW). We observed slightly reduced JA accumulation in mkp1, 149.8 ± 104.2 pmol/g FW in unwounded plants and increased to 3169.6 pmol/g FW in wounded plants,
although the differences from only two biological replicates were not statistically different from JA accumulation in Col-0 plants (Figure 3.2). Incidentally, some of the MAPK phosphatase mutants showed increased susceptibility when exposed to fungal gnat larvae (*Bradysia*, sp.). This opportunistic herbivore normally does not cause damage to Col-0 plants (McConn et al., 1997). The accidental exposure of plants to fungal gnat larvae resulted in greater damage to *phs1*, *abi1*, and *dsptp1* plants compared to Col-0 and the other phosphatase mutants (Figure 3.3). This indicates that the loss of individual MAPK phosphatases leads to reduced resistance against an herbivore.
Figure 3.2: JA accumulation at 1 h after wounding is not altered in MAPK phosphatase mutants. MAPK phosphatase mutant plants were wounded with hemostats twice parallel to the midvein. Unwounded rosette leaves were collected immediately before wounding. Wounded rosette leaves were collected at 60 mins following hemostat wounding. JA levels were determined by vapor phase extraction, followed by GC-MS. Bars represents the percent difference of the average JA levels in MAPK phosphatases compared to Col-0 ± standard deviation of 2-4 biological replicates. JA levels in all mutants were not significantly different from Col-0.

Figure 3.3: Increased susceptibility in MAPK phosphatase mutants to fungal gnat larvae. Arabidopsis plants were grown in soil under long day conditions. Damage to susceptible mutant plants began ~5 to 6 weeks after germination. Image shows feeding larva on a phs1 rosette leaf. Image is representative of damage caused by feeding larvae on phs1, abi1, and dsptp1. The larvae did not attack other mutants or Col-0.
Role of MAPK phosphatases during PAMP-triggered MAPK signaling

*Arabidopsis* AP2C1, PP2C5, and MKP1 are involved in the regulation of PAMP-mediated immune responses (Schweighofer et al., 2007; Brock et al., 2010; Anderson et al., 2011). To assess the role of phosphatases during PAMP-triggered immune responses, *Arabidopsis* MAPK phosphatase mutants were exposed to the bacterial elicitor, flg22. This elicitor rapidly activates MPK6, MPK3 and MPK4, with the peak activation between 5 to 15 minutes (Nühse et al., 2000). *Arabidopsis* MAPK phosphatase mutants *mkp1*, *ibr5*, and *dsptp1* demonstrate increased accumulation of active MPK6 compared to Col-0 after 30 min of flg22 elicitation (Figure 3.3). We also observed greater accumulation of phosphorylated MPK6, MPK3, and possibly MPK4 in *dsptp1* at 5 mins, and stronger phosphorylation of MPK3 at 5 min in *mkp1*, compared to Col-0 and the other mutant lines. Taken together, MAPK phosphatases are involved in the dephosphorylation of MPK6 and MPK3 during flg22-triggered immune responses.

Plants stimulate SA biosynthesis and accumulation during PAMP-triggered and avirulent-induced defense responses (DebRoy et al., 2004). Since SA biosynthesis may be regulated by MAPK signaling (Zhang et al., 2007b), we measured the accumulation of SA in the same unwounded and wounded MAPK phosphatase mutant plant samples that were used for the measurement of JA. In all unwounded and wounded MAPK phosphatase mutants, SA accumulation was unaltered compared to control plants (Figures 3.5A and 3.5B). Wounding is known to cause rapid JA accumulation, however, there is only one report that observed a transient increase in SA accumulation following wounding, which began increasing 3 hours after wounding and peaked between 6 and 24 hours after wounding (Ogawa et al., 2010). We did not observe altered SA accumulation
in the MAPK phosphatase mutants, which indicates the MAPK phosphatases do not regulate basal SA levels or SA accumulation in response to wounding, but are involved in regulating MAPK activity following activation by the bacterial elicitor, flg22.

Figure 3.4: Loss of MAPK phosphatase mutants lead to prolonged MPK6 activation in response to flg22. Leaf disks were excised from Col-0 and MAPK phosphatase mutants and treated with 10 nM flg22 for 0, 5, or 30 minutes. Samples were analyzed for MAPK activity as described in Figure 3.1. Immunoblots were probed with anti-pERK. Each sample represents total protein extracted from eight 4 mm leaf disks collected from 4 different plants. Immunoblot was stained with coomassie to show protein loading (CBB).

Figure 3.5: SA Accumulation is not altered in MAPK phosphatase mutants. MAPK phosphatase mutants were wounded and collected in the same manner as described for Figure 3.2. A) Data represents the mean percent difference of SA accumulation in unwounded (light gray bars) and B) wounded (dark gray bars) MAPK phosphatase mutants compared to unwounded and wounded Col-0, respectively. The mean SA accumulation in Col-0 unwounded and wounded is set to 100%. Error bars represent the ± standard deviation of 2-3 biological replicates. (Except pp2c5 in Figure 3.3A, which represents one replicate).
Discussion

Protein phosphorylation is one of the most common post-translational modifications in eukaryotes. MAP kinase phosphorylation cascades are central hubs in the signaling and regulation of many cellular functions and are conserved in all eukaryotes. In plants, MAPK pathways have been well studied, however, very little is know about the regulation of MAPKs by MAPK phosphatases. Currently, there is little know regarding the role of MAPK phosphatases in the regulation of MAPKs in response to herbivory and only limited evidence of MAPK phosphatase involvement in regulating the MAPK response to pathogens. Our preliminary data suggests novel roles of some MAPK phosphatases in the regulation of *Arabidopsis* MAPK signaling in response herbivory and pathogen infection.

MAPK phosphatase T-DNA mutant lines subjected to mechanical wounding and analyzed for MAPK activity showed altered MAPK activity. There is no prior evidence of MAPK signaling regulation by MAPK phosphatase in response to wounding or herbivory. We observed prolonged wound-induced MPK6 and MPK3 activity in *ap2c1, abi1, dsptp1, phs1*, and *pp2c5*. In most cases, MPK6 and MPK3 are highly regulated and under tight control. Studies in animals show that the duration and amplitude of MAPK signaling is important for the specific output response, e.g. cell differentiation versus proliferation (Mayawala et al., 2004; Murphy et al., 2004; Murphy and Blenis, 2006). To determine if the altered MAPK activity caused an altered physiological response downstream of MAPK signaling, we measured the accumulation of JA in plants one hour after wounding. We hypothesized that prolonged MAPK activity would result in altered JA accumulation, however, we observed no significant change in JA accumulation in
mutants that demonstrated prolonged MAPK phosphorylation. A previous study of ap2c1 found an approximately 4-fold increase in JA accumulation four hours after wounding compared to Col-0 plants (Schweighofer et al., 2007). The difference between this and our analysis may be due to the time when the samples were taken. Our future studies will need to examine JA levels over at least four hours or an even longer period of time. Additionally, some MAPK phosphatase mutants were more susceptible to damage from gnat larvae feeding. A prior study by McConn et al., 1997, found that Arabidopsis mutants deficient for the JA precursor, linolenic acid, were unable to produce JA and were much more susceptible to fungal gnat larvae. This indicates that the phosphatase mutants may exhibit a yet unknown deficiency in JA synthesis or signaling. While MPK6 orthologs in tobacco and tomato regulate JA synthesis and are not activated by JA (Kandoth et al. 2007; Stratmann and Ryan, 1997), one report shows that JA can activate MPK6 in Arabidopsis, indicating a role for MPK6 in JA signaling (Takahashi et al., 2007).

MAPK phosphatases exposed to the bacterial elicitor flg22 also demonstrated altered MPK6 and MPK3 activity in mkp1, ibr5, pp2c5, and especially in dsptp1 mutant plants. Although an extended time course similar to the wounding experiment (Figure 3.1) was not obtained in response to flg22, the number of mutants that affect flg22-induced MAPK activity indicates that multiple phosphatases regulate MAPK activity in response to various stimuli. Results for mkp1 confirm an earlier study, which demonstrated stronger MPK3 and MPK6 activity between five and ten minutes after elicitation by the PAMP, elf26 (Anderson et al., 2011). Our collaborator Dr. Zhengqing Fu (University of South Carolina) tested MAPK phosphatase mutants for resistance
against a bacterial pathogen and found that \textit{pp2c5} and \textit{dsptp1} plants were more resistant to \textit{Pseudomonas syringe pv. maculicola} 4326. Although some MAPK phosphatase mutants were more resistant to \textit{P. syringae pv. maculicola} 4326, we did not observe altered SA accumulation in unwounded or wounded mutant plants (Figure 3.5). Further research is necessary to test all of the available mutants for altered MAPK signaling over a longer period of time. Additionally, it will be important to correlate altered MAPK signaling with altered downstream responses, such as PAMP-induced ROS production, SA accumulation and signaling in response to a pathogen, and altered defense gene expression.

In Chapter 2, we demonstrated that MAPKs associate with a multi-protein complex, which may be involved in the dephosphorylation of MAPKs. This indicates that MAPK phosphatases may associate with this complex or scaffolding protein and contribute to the regulation of immune responses to herbivory and pathogens. Further research is needed to evaluate the association of the 11 known MAPK phosphatases with a MAPK protein complex, starting with the phosphatases whose loss caused the greatest differences in MAPK activity (e.g. AP2C1 and DsPTP1).

In both the response to wounding and to flg22, the MAPK activity returned to or began to decrease to basal levels after the initial peak of MAPK activity. This indicates that more than one phosphatase is involved in the dephosphorylation of MAPKs during plant immune responses. To further examine the role of MAPK phosphatases, it will be important to generate double MAPK phosphatase mutants. It may be necessary to generate triple or higher level mutants, depending on the extent of overlapping regulation. Currently, crosses between all 7 confirmed homozygous phosphatase mutants have been
completed, and we are currently establishing homozygous double mutant lines from the resulting 21 possible crosses. We have successfully generated the \textit{dsptp1}/\textit{mkp1} mutant line and will begin testing it soon.

Together, our results demonstrate novel roles of MAPK phosphatases in the regulation of mechanical wounding- and bacterial elicitor-induced MAPK activity. This work will serve as the basis for a more detailed analysis of MAPK phosphatase function in defense responses.
Summary of Research Goals and Results

The aim of my doctoral studies was to further characterize the roles of MAP kinases in signaling responses to biotic stress. This dissertation focuses on three different aspects of MAP kinase signaling in plants: 1. Role of tomato MAPKs MPK1, MPK2, and MPK3 in programmed cell death, 2. Identification and characterization of a MAP kinase-containing multi-protein complex, 3. Regulation of MAPK signaling by MAPK phosphatases. This research is explores how a few MAPKs act as central hubs in the cellular stress signaling network and how regulation of MAPK signaling affects cellular stress responses.

Although prior studies have shown that overexpression of MAPKs can lead to cell death (Yang et al., 2001; Zhang and Liu, 2001; Ren, et al., 2002), we show here that when tomato MPK1/2/3 are co-silenced, it causes severe spontaneous, runaway cell death. This suggests that MAPKs play an important role in the negatively regulate programmed cell death. Since overexpression and silencing of MAPKs can lead to cell death, MAPK homeostasis seems to be vital to cells. In Chapter 1, the runaway cell death phenotype is examined using protein and gene expression analysis, measurement of plant hormone changes, microscopic characterization of lesion areas, and the effect of silencing on the oxidative burst response to bacterial elicitors. MPK1/2/3 silencing, but not MPK1/2 or MPK3 silencing alone, causes formation of lesions that correspond to areas with the strongest silencing and interestingly, areas of catalase protein accumulation. The severity of the lesion phenotype appears to be dependent on the degree of MPK3
silencing in combination with MPK1/2 silencing. Using two VIGS silencing vectors, which only vary in the sequence of MPK3 used as a silencing target, we see a much more severe and stunted phenotype in plants with stronger MPK3 reduction. Additionally, *MPK1/2/3*-silenced plants have reduced JA accumulation in response to mechanical wounding and increased accumulation of SA compared to control plants. Since there is a correlation between lesion formation and catalase protein accumulation, we examined ROS levels and the oxidative burst response to the bacterial elicitor, flg22. Although basal levels of ROS are unaffected by *MPK1/2/3*-silencing, areas with lesions have an increased accumulation and production of ROS during oxidative bursts induced by flg22. Although the specific mechanism leading to the formation of lesions is not known, this research shows an interesting additional role of MAPKs and a potential interaction between MAPKs and catalase in the regulation of cell death.

In Chapter 2, we show that some MAPKs and a MAPKK associate with a high molecular weight multi-protein complex in tomato, tobacco, and *Arabidopsis*. Additionally, we show that the protein complex plays a role in the phosphorylation status of the MAPKs that associate with the complex. It had not been shown in plants that MAPK/MAPKKs are organized in large complexes, much like animals and fungi, where MAPK complexes are assembled on scaffold proteins. No MAPK scaffold proteins are known in plants, but our findings indicate that novel MAPK scaffolding proteins that lack homology to animal/fungal proteins may be present in plants and play a role in the dephosphorylation and regulation of MAPK signaling dynamics. A strategy was devised to identify additional constituents of this complex.
Chapter 3 provides a general characterization of MAPK phosphatases and their role in the regulation of MAPKs in *Arabidopsis*. The goals were to determine if MAPK phosphatases interact with MAPKs in the protein complexes identified in Chapter 2, and provide a systematic characterization of MAPK phosphatase functions in stress responses. So far, we did not identify any MAPK phosphatases associating with the protein complex. We found that the loss of MPK phosphatases alters of MPK6 and MPK3 activity in response to wounding and a bacterial elicitor. Additionally, we observed altered responses to a pathogen and a herbivore in some *Arabidopsis* MAPK phosphatase null mutants. This shows that disturbing proper MAPK signaling by manipulating MAPK inactivation results in altered responses to biotic stressors.
References


Bown, A.W., Hall, D.E., MacGregor, K.B. (2002). Insect footsteps on leaves stimulate the accumulation of 4-aminobutyrate and can be visualized through increased


jasmonic acid as intermediates in the octadecanoid signalling pathway. FEBS letters, 377, 523-529.


Theodoulou, F., Job, K., Slocombe, S., Footitt, S., Holdsworth, M., Baker, A., Larson, T., Graham, I. (2005). Jasmonic acid levels are reduced in COMATOSE ATP-


Yan, Y., Borrego, E., Kolomiets, M. V. (2013). Jasmonate biosynthesis, perception and function in plant development and stress responses. INTECH Open Access Publisher.


