DISSECTING MITOGEN-ACTIVATED PROTEIN KINASE CASCADES INVOLVING ARABIDOPSIS MKK6

by

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ABSTRACT

Mitogen-activated protein (MAP) kinase cascades act as critical components in the signalling pathways of all eukaryotic cells. They play a pivotal role in the transduction of extra- and intra-cellular stimuli and regulate cell growth, proliferation, differentiation and cell death, through sequential activation of MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MKKs), and MAP kinases (MPKs). These three components form modules that control the phosphorylation of various substrates including transcription factors, enzymes, and cytoskeleton-associated proteins. In the Arabidopsis genome, over 60 MAPKKKs (AtMKKK), 10 MAPKKs (AtMKK), and 20 MAPKs (AtMPK) have been identified. The smaller number of AtMKKs suggests that diverse signals may converge and be integrated at the level of AtMKK. Among the ten AtMKKs, MKK6 has been proposed to play a role in regulating cytokinesis. However, little is known about the hierarchal phosphorylation system containing MKK6.

In this Ph.D. project, I aimed to dissect the MAP kinase cascades involving MKK6 in Arabidopsis. I investigated potential targets of MKK6. Four MAP kinases were identified to interact with, and be phosphorylated by, MKK6, namely, MPK4, MPK6, MPK11, and MPK13. Among them, MPK13 is developmentally co-expressed with MKK6, and both MPK13 and MKK6 display high Promoter::GUS activity at the primary root tips and at the lateral root primordia. Partial
suppression of either MKK6 or MPK13 expression significantly reduces the number of lateral roots in the transgenic lines, suggesting that the MKK6-MPK13 module positively regulates lateral root formation.

Loss-of-function mutants of another potential target of MKK6, MPK4, are severely affected. In mpk4 mutant plants, anthers can develop normal microspore mother cells (MMCs) and peripheral supporting tissues, but the MMCs fail to complete meiotic cytokinesis, a phenotype reminiscent of those observed in both atnack2/tes/stud and anq1/mkk6 mutants. Biochemical analysis defines a putative signalling module linking AtNACK2/TES/STUD with ANP3, MKK6 and MPK4, suggesting a model in which the AtNACK2-ANP3-MKK6-MPK4 cascade specifically facilitates male meiotic cytokinesis in Arabidopsis.

In summary, my PhD work has expanded our understanding of the diversity of MAP kinase cascades. Different MPKs can act as downstream targets of the same MKK with tight spatial and temporal regulation during plant development.
PREFACE

A version of Chapter 3 has been resubmitted and is under revision. [Zeng, Q.], Chen, J-G. and Ellis, B.E. (2010) **MPK4 is required for male-specific meiotic cytokinesis in Arabidopsis.** Chen J-G, Ellis B.E. and I initiated the project and designed the experiments. I conducted all the experiments and wrote the manuscript. Ellis B.E. revised the manuscript.
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<table>
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<th>Description</th>
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<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>6-BA</td>
<td>6-benzyladenine</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylate</td>
</tr>
<tr>
<td>AOA</td>
<td>aminooxyacetic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BiFC</td>
<td>bi-molecular fluorescence complementation</td>
</tr>
<tr>
<td>BL</td>
<td>brassinolide</td>
</tr>
<tr>
<td>CA</td>
<td>constitutively active</td>
</tr>
<tr>
<td>CaMV35S</td>
<td>Cauliflower Mosaic Virus 35S (constitutive) promoter</td>
</tr>
<tr>
<td>CBB</td>
<td>coomassie brilliant blue</td>
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<td>cDNA</td>
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<tr>
<td>CDS</td>
<td>coding sequence</td>
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<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>cYFP/nYFP</td>
<td>C-terminus/N-terminus of yellow fluorescent protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEX</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ET</td>
<td>ethylene</td>
</tr>
<tr>
<td>EV</td>
<td>empty vector</td>
</tr>
<tr>
<td>GA₃</td>
<td>gibberellic acid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
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<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
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<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>Term</td>
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<tr>
<td>-------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>bp</td>
<td>bases pair</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani bacterial growth medium formula</td>
</tr>
<tr>
<td>MAPK (or MPK)</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKK (or MKK)</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MKP</td>
<td>mitogen-activated protein kinase phosphatase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>NAA</td>
<td>naphthalene-1-acetic acid</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription-polymerase chain reaction</td>
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<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>S.E.</td>
<td>standard error</td>
</tr>
<tr>
<td>SC</td>
<td>synthetic complete</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TAIR</td>
<td>The Arabidopsis Information Resource</td>
</tr>
<tr>
<td>T-DNA</td>
<td>transfer DNA</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
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<td>X-gluc</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
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<tr>
<td>Y2H</td>
<td>yeast two-hybrid</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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First of all, I would like to thank my supervisors Brian Ellis and Jin-Gui Chen for providing me the opportunity to pursue my PhD degree under their supervision. Brian has inspired me through his wisdom in academy and life, passion for science and education, and encouragement and patience in my learning process. I thank Jin-Gui for his careful and detailed guidance especially in the early years of my program, when I just started to taste research life. His incredible efficiency and enthusiasm about science challenged me to improve my research ability and scientific quality. They have been amazing mentors in training me and helping me getting this degree.

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CHAPTER 1 INTRODUCTION
1.1 Introduction to MAP Kinases

1.1.1 Canonical model of a MAP kinase cascade

Protein phosphorylation by protein kinases is one of the principal regulatory events by which proteins are post-translationally modified. Among the eukaryotic protein kinases, mitogen-activated protein (MAP) kinases represent a distinctive and highly conserved protein kinase sub-family (Hamel et al., 2006). The canonical model of MAP kinase cascades is a three-tiered module conserved through all eukaryotic cells that have been studied to date. MAP kinase kinase kinases (MAPKKKs), the initial component in the MAP kinase cascade, are serine/threonine protein kinases which are activated by a MAPK kinase kinase kinase (MKKKK) or by interaction with Ras or Rho GTP-binding proteins (Widmann et al., 1999). The activated MAPKKKs phosphorylate specific serine and/or threonine residues in the activation loop (T-loop) of the MAP kinase kinases (MKKs). Once activated, the dual-specificity MKKs phosphorylate MAP kinases on threonine and tyrosine residues (also called the TXY motif) located on the activation loop between the conserved kinase subdomains VII and VIII (Bardwell and Shah, 2006). The dual phosphorylation causes conformational changes in the kinases, which thereby become fully active (Zhang et al., 1994).

MAP kinases are serine/threonine kinases that phosphorylate a consensus Ser/Thr-Pro motif, or the more stringent Pro-Xaa-Ser/Thr-Pro motif, of a variety of downstream targets, such as transcription factors, enzymes, and microtubule-associated proteins (Chen and Thorner, 2007, Gonzalez et al., 1991, Nakagami et al., 2005). Through these sequential phosphorylation events within the linear cascades consisting of at least three kinase components, cells are able to amplify and direct various input signals to downstream effectors, leading to the corresponding biochemical and physiological modifications in response to such stimuli.

The inactivation of a MAP kinase is achieved by protein phosphatase-catalyzed dephosphorylation, which removes the phosphate group(s) from either the threonine residue or the tyrosine residue, or both (Theodosiou and Ashworth,
Although both serine/threonine phosphatases and tyrosine phosphatases have been reported to deactivate MAP kinases, the name ‘MAP kinase phosphatase’ (MKPs) refers to a specific group of dual-specific phosphatases (DSPs) that are thought to be specialized for dephosphorylation of the pTXpY motif in activated MAPKs. The intensity and duration of the output signal that is channeled through a MAP kinase cascade is thus spatially and temporally modulated by the interplay between MAPK kinases and MAPK phosphatases.

1.1.2 MAP kinases in mammalian and yeast cells

Early in the 1980s, it was noticed that cell growth factors can rapidly stimulate the tyrosine phosphorylation of a 42kDa protein (Cooper et al., 1982). This phosphoprotein is itself a serine-threonine protein kinase that phosphorylates microtubule-associated proteins (MAP), and therefore was termed a MAP kinase (Ray and Sturgill, 1988). It was soon realized that the MAP kinase could be activated by a variety of mitogens, including epidermal growth factor, platelet-derived growth factor, and insulin, and that phosphorylation of the kinase on both tyrosine and threonine residues was required for its enzyme activity (Ray and Sturgill, 1988, Rossomando et al., 1989). It also became apparent that the MAP kinase was capable of phosphorylating substrates other than microtubule-associated proteins, and hence the term ‘MAP kinase’ now serves as a contraction of the descriptor, “mitogen-activated protein kinase”.

Nearly thirty years of research has enormously expanded our understanding of MAP kinase signalling pathways. In mammalian cells, MAP kinases comprise several subgroups, designated the conventional Extracellular signal-Regulated Kinases (ERK1 and ERK2), Jun N-terminal kinases (JNK1, JNK2 and JNK3), p38 kinase isozymes (p38α, p38β, p38γ and p38δ), ERK5, as well as the unconventional ERK3/4, Nemo-like kinase (NLK), and ERK7 (Coulombe and Meloche, 2007, Qi and Elion, 2005). ERK1 and ERK2 are the best described MAPKs in mammalian cells, where they are activated by many stimuli, including growth factors, cytokines, virus infection, and ligands for G protein-coupled receptors, and participate in regulation of meiosis, mitosis, and postmitotic
functions (Johnson and Lapadat, 2002). ERK1/2 MAP kinases contain a Thr-Glu-Tyr motif that is phosphorylated by the active MAPKKs, MEK1/2 (Whitmarsh and Davis, 1996). Members of the JNK and p38 subfamilies are mainly activated in response to various stresses, and contain a Thr-Pro-Tyr and Thr-Gly-Tyr tripeptide activation loop motif, respectively (Derijard et al., 1994, Raingeaud et al., 1995). ERK5 (also called Big MAPK1 (BMK1)), which possesses the Thr-Glu-Tyr activation motif and a large C-terminal extension, is activated by epidermal growth factor (EGF), osmotic stresses, oxidative stresses, and G-proteins/G protein-coupled receptors (Abe et al., 1996, Lee et al., 1995, Obara and Nakahata, 2010). The atypical MAP kinases, ERK3 and ERK4, possess a single phospho-acceptor site (Ser-Glu-Gly) in the activation loop instead of the conventional Thr-Xaa-Tyr motif (Turgeon et al., 2002). Another two atypical MAP kinases, NLK (Thr-Gln-Glu motif) and ERK7 (Thr-Glu-Tyr motif), are also considered to belong to the mammalian MAP kinase family (Abe et al., 2001, Abe et al., 1999, Brott et al., 1998).

Yeast (Saccharomyces cerevisiae) MAP kinases are well defined and can be positioned within five signalling pathways (Chen and Thorner, 2007). The MAP kinase cascade regulating the yeast mating pheromone response consists of Ste11 (MKKK), Ste7 (MKK), and Fus1 and Kss1 (MPKs). Both MPKs contain a Thr-Glu-Tyr motif, which is analogous to mammalian ERK7 (Bardwell, 2004, Wang and Dohlman, 2004). In addition to participation in mating pheromone responses, Kss1 is required for filamentous growth under nutrient limitation conditions (Truckses et al., 2004). Another MAP kinase, Hog1 (Thr-Gly-Tyr motif), is critical for yeast survival under the high osmolarity glycerol (HOG) growth conditions (O'Rourke et al., 2002, Saito and Tatebayashi, 2004). The fourth MAP kinase, Slt2/Mpk1 (Thr-Glu-Tyr motif) contributes to the maintenance of cell wall integrity (CWI) and budding (Levin, 2005). Finally, the fifth MAP kinase, Smk1 (Thr-Xaa-Tyr) is only expressed during sporulation in response to carbon and nitrogen deprivation, and is specifically required for the spore-specific cell wall layer assembly during meiosis (Krisak et al., 1994, Neiman, 2005, Pierce et al., 1998).
1.1.3 Signalling specificity and regulatory mechanism

The signalling specificity of MAP kinase cascades is enhanced by effective and precise binding of MAP kinases to their interacting proteins, i.e., the activating MAPK kinases, the inactivating phosphatases, and the MAP kinase substrates. The binding site of the MAP kinases is localized in the C-terminal region of the MAP kinase primary sequence, and contains a group of negatively charged amino acids. This site confers specific recognition of the MAP kinase partners, including the regulators and the substrates (Tanoue et al., 2000). This site is referred to as the CD domain, or the common docking domain. In addition, the ED site, which is close to the CD domain on the protein surface, enhances the specificity of the substrate binding in the mammalian p38 MAP kinases. This corresponds to the TT site in ERK2 (Tanoue et al., 2001).

On the other hand, MAPK-docking sites can also be identified in the MAPK-interacting proteins. The D-domain (DEJL motif), can be identified in many MAPK-interacting proteins, and contains a conserved \((K/R)_{1-3}\cdot X_{1.5}\cdot \Phi_A X \Phi_B\) sequence, where \(\Phi_A\) and \(\Phi_B\) are hydrophobic residues (Yang et al., 1998). A second characterized docking motif has a consensus \(L\cdot X_{1.2}\cdot (R/K)_{2.6}\) (Smith et al., 1999). Both of these consensus sequences possess positively charged amino acids that can bind to the negatively charged amino acids in the CD domain of MAPKs. A third docking site on MAPK-interacting proteins is named the DEF motif and has a consensus sequence FXFP (Jacobs et al., 1999). Together, these docking motifs facilitate efficient enzyme-substrate selection and are critical to the pathway regulation.

In addition to the three classes of functional interactors mentioned above, non-catalytic scaffold proteins make essential contributions to MAP kinase signalling specificity by providing local activation platforms that insulate different MAP kinase pathways. In yeast, the Ste11 MAPKKK participates in three distinct MAP kinase-mediated signalling pathways: mating, filamentation, and high-osmolarity glycerol response (Gustin et al., 1998, van Drogen and Peter, 2002). In response to pheromone signals, Ste11 is recruited by Ste5, a scaffold protein that tethers
multiple kinases operating in the mating pathway (Choi et al., 1994). However, when bound by another scaffold protein, Pbs2 (a MKK), Ste11 is controlled by the osmosensor Sho1 in the response to high osmolarity (Widmann et al., 1999). Further studies showed that the scaffolds are not only flexible organizers that effectively tether signalling components together (Harris et al., 2001, Park et al., 2003), but also could be active players that change the configuration of a protein to be a more efficient substrate for a certain kinase (Good et al., 2009). Therefore, the scaffolds recruit different combinations of components to make them dedicated to certain pathways. In mammalian cells, scaffold proteins can bind to MAP kinases and enhance kinase activities such as MP1, (Schaeffer et al., 1998) or inhibit them, as in the case of JIP-1, (Whitmarsh et al., 1998). Finally, the scaffolds can be the MAPK module components, such as mammalian MEKK1, a MAPKKK (Xu and Cobb, 1997), and the yeast Pbs2, a MKK (Posas and Saito, 1997).

The activation of a MAPK by MAPK kinase is often accompanied by the physical release of the MAPK from the MAPK:MPK kinase complex, which leaves the MPK docking motif available to bind with its substrates and with corresponding MKPs (Avruch, 2007). In addition, such disassociation and phosphorylation of the target proteins can lead to cellular compartmentalization of the signalling components. In some cases, the kinases translocate from the cytoplasm to the nucleus, and this transportation requires nuclear import factors (O'Rourke et al., 2002). In other cases, the MAP kinase module moves from the cytoplasm to the plasma membrane to serve as a molecular switch operating in proximity to the site of the incoming signal(s). Interestingly, the activation threshold for signal transmission through the module can be much lower when associated with the plasma membrane than when localized in the cytosol (Harding et al., 2005). In short, the subcellular localization of the MAP kinase components contributes to the proper execution of the signalling transduction.

Positive and negative feedback loops are common features of signalling pathways, and this is also true in the MAP kinase cascades. Recently, it was reported that the mammalian MKK, MEK1, forms heterodimers with the other
MKK, MEK2, and this interaction negatively regulates the MEK2-dependent ERK signalling (Catalanotti et al., 2009). This new regulatory mechanism adds another level of complexity of the MAP kinase phosphorelay.

1.2 MAP Kinases in Plants

The first plant MAP kinase gene was cloned from the genomic DNA of *Brassica napus* (Stafstrom et al., 1993). Since then, many studies have contributed to unveiling the complexity of MAP kinase cascades across many plant species, including alfalfa (*Medicago sativa*), tobacco (*Nicotiana tabacum*), Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), poplar (*Populus trichocarpa*), tomato (*Lycopersicon esculentum*), maize (*Zea mays*), cotton (*Gossypium hirsutum*) and parsley (*Petroselinum crispum*). These reports have demonstrated the importance of plant MAP kinase modules in regulating both environmental and developmental signalling response pathways.

To date, all known plant MAP kinases (MPKs) contain either a TEY or TDY motif in their activation loop, and therefore are considered to belong to the mammalian ERK subfamily (Ichimura et al., 2002). Genome-wide searches in plants have not uncovered homologues of other mammalian or yeast MAP kinase subfamilies in plants. In addition, plant MPK kinases (MKKs) possess unique phosphorylation sites. Instead of having the consensus sequence S/TxxxS/T that is found in mammalian MKKs, plant MKKs contain a more extended S/TxxxxxS/T sequence in their kinase domain, located at the C-terminus of the protein. The sequenced genome of the model dicot plant, *Arabidopsis thaliana*, encodes over 60 MAPKKKs (AtMKKK), as well as 10 MAPKKs (AtMKK), and 20 MAPKs (AtMPK) (Ichimura et al., 2002). The completed poplar (*Populus trichocarpa*) and rice (*Oryza sativa*) genomes reveal that they contain 9 *PtMKKs*, and 21 *PtMPKs*, and 8 *OsMKKs*, and 15 *OsMPKs*, respectively (Hamel et al., 2006). It is notable that, even though the plant MAP kinases belong only to the ERK subfamily, they consistently outnumber the mammalian MAP kinases. It is also notable that there are always fewer MKKs than MPKs in plants (Rodriguez et al., 2010). However, unlike mammalian ERKs, whose functions are controlled primarily by
extracellular signals, plant MAP kinases are not functionally limited to responding to growth factors. Instead, the expanded plant MAP kinase family components form an integrated complex signalling network and they seem to be involved in a broader range of pathways, including stress responses and pathogen defense, hormonal and developmental signalling, cell cycle control and cytoskeleton regulation (Andreasson and Ellis, 2010, Rodriguez et al., 2010).

1.3 MAP Kinase Cascades in Arabidopsis

Over 60 MAPKKKs have been identified in the Arabidopsis genome, and they belong to two distinct clades: the MEKK (or STE) superfamily and the raf superfamily (Champion et al., 2004). Strikingly, downstream of the MAPKKKs, there are only ten MKKs in Arabidopsis, suggesting that multiple signals must converge on, and presumably be integrated at, the MKK level. The activated MKK(s) would then precisely recruit those specific downstream MPKs needed to modify the appropriate MPK substrates (Andreasson and Ellis, 2010). Large scale analyses of the MAP kinase family members have been performed in order to identify novel MKK-MPK cassettes and develop a comprehensive picture of the plant MAP kinase network. By analyzing available microarray data, Menges et al. (2008) generated a comprehensive overview of gene expression patterns for 114 MAP kinases, including all AtMPKs, AtMKKs, and most AtMKKKs, and extracted predictions of potential MAP kinase cascades based on co-expression of particular kinases in certain biological contexts (Menges et al., 2008). Hua et al tested the efficacy of nine constitutively activated AtMKKs (MKK1 to MKK9) in phosphorylating 11 of the 20 AtMPKs, which were transiently co-expressed in leaf tissue of Nicotiana benthamiana (Hua et al., 2006). A protein microarray including 1,690 Arabidopsis proteins derived from an inflorescence meristem cDNA library was generated and used to identify potential substrates of MPK3 and MPK6, two important MPKs that are engaged in both developmental program and stress and disease signalling (Feilner et al., 2005). A similar but much broader study identified known and novel MKK-MPK modules by analyzing the functional relationships between nine AtMKKs and ten AtMPKs. It generated
a list of putative MPK targets by employing protein microarrays carrying 2158 recombinant Arabidopsis proteins (Popescu et al., 2009). A directed yeast two-hybrid assay was conducted to test the pair-wise interactions between the ten MKKs and all 20 MPKs in the Ellis lab and the positive interactions were followed up with in vitro kinase activity assays (Lee et al., 2008). Depending on the methods the various studies used, different screens have uncovered sets of MKK-MPK interaction/phosphorylation events that sometimes, but not always, overlap (Table 1.1). Some of the predicted cascades have been experimentally tested either biochemically or genetically, while other potential models are novel and remain to be confirmed. The following sections summarize the published studies of the members of the four sub-groups of Arabidopsis MKKs and review our current understanding of the functions of each AtMKK, with respect to their activators and targets within particular biological contexts.
Table 1.1 Connections of Arabidopsis MPKs with MPK kinases (MKKs)

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◊ Hua et al., 2006
□ Popescu et al., 2009
◆ Y2H ○ in vitro kinase assay Lee et al., 2008
1.3.1 Functional characteristics of the Arabidopsis MKKs

Group A MKKs

The Group A MKKs comprise three members, namely, MKK1, MKK2, and MKK6. MKK1 and MKK2 are 68% identical at the amino acid level, and form a paralogous gene pair, while MKK6 is relatively distinct. A MAP kinase cascade involving MEKK1-MKK1/MKK2-MPK4 was identified in the early days of plant MAP kinase studies, through protein-protein interaction assays (Huang et al., 2000, Ichimura et al., 1998). Genetic analysis established that MPK4 negatively regulates systemic acquired resistance (SAR), probably through its influence on salicylic acid accumulation (Petersen et al., 2000). The MAPKKK, MEKK1, has been positioned upstream of MPK4 in ROS homeostasis (Nakagami et al., 2006), in tissue type- and temperature-dependent cell death (Ichimura et al., 2006), and in flg22-induced disease resistance response (Suarez-Rodriguez et al., 2007). Intriguingly, unlike either the mekk1 or mpk4 mutants, mkk1 null mutants are more susceptible to both the virulent and avirulent isolates of Pseudomonas syringae pv. tomato DC3000, while mkk2 plants display no dramatic difference from WT upon treatment (Brader et al., 2007, Meszaros et al., 2006). The roles of MKK1 and MKK2 had remained unclear until the double mutant mkk1/mkk2 was generated; it displayed constitutive Pathogenesis-Related (PR) gene expression and enhanced disease resistance (Gao et al., 2008, Qiu et al., 2008b), suggesting that the closely related MKK1 and MKK2 paralogues share redundant function in regulating disease response, apparently while linking MEKK1 and MPK4. In addition to their overlapping function in plant immunity, however, MKK1 and MKK2 also have separate functions. Cold and salt stresses activate both MPK4 and MPK6, and their activation is impaired in the mkk2 null mutants, which are hypersensitive to these stresses, suggesting that MKK2 is specifically required for cold and salt tolerance in plants (Teige et al., 2004). The MKK1-MPK6 cascade is indispensible for H$_2$O$_2$ (ROS) production and for ABA-induced expression of CAT1, which encodes a major ROS-scavenging enzyme (Xing et al., 2008). Later, it was shown that both MKK1 and MPK6 positively regulate the
ABA and sugar signalling in non-stratified seed germination (Xing et al., 2008, Xing et al., 2009).

MKK6, which is in subgroup A2, is proposed to play a role in cytokinesis based on both the mkk6 knockout mutant phenotype and the functional analysis of its tobacco orthologue, NtNQK1 (Soyano et al., 2002). It will be discussed in detail later.

**Group B MKKs**

Arabidopsis, along with all other higher plants examined, appears to possess a single Group B MKK (Hamel et al. 2006). MKK3 contains an unusual ‘nuclear transport factor 2’ (NTF2) domain in its structure and is phylogenetically distinct from other MKKs, forming a single gene clade (group B). Shinozaki’s group reported that MKK3 can phosphorylate MPK6 and that this MKK3-MPK6 module negatively regulates jasmonic acid (JA) inhibition of Arabidopsis seedling growth (Takahashi et al., 2007). Another research group identified a set of group C MPKs, namely, MPK1, MPK2, MPK7, and MPK14, as interactors of MKK3 (Doczi et al., 2007). The authors found that mkk3 mutant plants were more susceptible to *Pseudomonas syringae* treatment, and that MKK3 over-expression lines were more resistant to the bacterial infection (Doczi et al., 2007). However, the activity of MPK7 is only induced by H₂O₂ treatment when co-expressed with MKK3, but not by flg22 application under the same condition (Doczi et al., 2007), suggesting that the MKK3-MPK7 module functions as a positive regulator of (probably) the secondary response of infection-induced oxidative burst (Rodriguez et al., 2010). Given that the C1 subgroup MAP kinases, MPK1 and MPK2, are activated by diverse stresses and hormone signals, including wounding, JA, ABA, and hydrogen peroxide (Ortiz-Masia et al., 2007), it would be interesting to examine whether MKK3 acts as the upstream activator of MPK1 and MPK2 in these stress responses.

**Group C MKKs**

Another two closely-related MKKs, MKK4 and MKK5, share 80% identity at the amino acid level and they comprise the group C MKKs. In most studies, MKK4
and MKK5 have been considered to be functionally equivalent. In addition, MKK4 and MKK5 both seem to be upstream activators of the paralogous gene pair, MPK3 and MPK6, in various pathways. An early study identified a MEKK1-MKK4/MKK5-MPK3/MPK6 cascade and placed it downstream of the receptor-like kinase flagellin receptor, FLS2, by using the Arabidopsis leaf mesophyll protoplast system (Asai et al., 2002). However, subsequent genetic and biochemical analysis provided evidence that while MEKK1 is required for flg22-induced activation of MPK4, it is not required for activation of MPK3 and MPK6 (Suarez-Rodriguez et al., 2007). Nevertheless, a large volume of experimental data does point to a role for MPK3 and MPK6 signalling in plant resistance to pathogen infection. Silencing of MPK6 was reported to compromise both basal and R-gene-mediated plant resistance (Menke et al., 2004). MPK3 and MPK6 also play a positive role in regulating fungal pathogen (Botrytis cinerea)-induced camalexin biosynthesis in Arabidopsis (Ren et al., 2008), and they are reported to be required for the full-priming of stress responses (Beckers et al., 2009). Both kinases are targets of the pathogenic bacterial effector, HopAI1 (Zhang et al., 2007a). In response to flg22, MPK6 phosphorylates and disassociates from the ethylene responsive factor 104 (ERF104), which is a key player in plant basal immunity (Bethke et al., 2009). However, it remains to be established whether the other MPK3/6-associated MKKs, MKK4 and MKK5, are required in the plant-pathogen interaction context. Very recently, it was reported that the Pseudomonas syringae type III effector HopF1 can interact with, inactivate, and ADP-ribosylate MKK5, and possibly MKK4 as well, events that effectively inhibit pathogen-associated molecular patterns (PAMP)-triggered plant immunity (Wang et al., 2010c). It will be a future challenge to define the roles of MKK1/MKK2, MKK4/MKK5, and MPK3/MPK6/MPK4 in PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI)-induced defense response and provide a clearer view of the PTI/ETI-associated signalling network.

Alongside the role(s) that MKK4 and MKK5 play in mediating biotic stress responses, they influence a number of aspects of plant development as well. The MKK4/MKK5-MPK3/MPK6 cassette operates downstream of YODA, a MAPKKK
essential for the establishment of the extra-embryonic suspensor cell (Lukowitz et al., 2004). They also negatively regulate stomata specification in the epidermis, since loss-of-function mutants display clustered stomatal patterning (Bergmann et al., 2004, Wang et al., 2007). This MAP kinase cascade is proposed to be the downstream of the two stomata cell lineage regulators, the membrane associated leucine-rich repeat receptor kinases ERECTA (ER) and TOO MANY MOUTHS (TMM) (Casson and Gray, 2008, Serna, 2009), while the output kinases of the cascade, activated MPK3/MPK6, phosphorylate the transcription factor SPEECHLESS (SPCH) on multiple sites (Lampard et al., 2008). SPCH is a positive regulator of stomatal development, and its phosphorylation subjects SPCH to proteolytic degradation (Lampard et al., 2008). In addition to that, both YODA and MPK3/MPK6 are required for proper ovule integument development, and therefore MKK4/MKK5 are likely to provide the links between the MAPKKK and MPKs (Wang et al., 2008a). Similarly, because mpk3+/mpk6- and er-105 erl1-2 erl2-1 (ER and ER-like) mutants displayed similar defects in anther lobe formation and anther cell differentiation, MKK4/MKK5 could be forming part of the underlying signalling pathway in this developmental process as well (Hord et al., 2008). However, that proposition is based solely on the phenotypic resemblance. Whether the MAP kinases are the genetic and molecular targets of ER-family members has not yet been defined. It would be worth examining whether the ER-family members are the upstream activators of YODA-MKK4/MKK5-MPK3/MPK6 cascade in both the stomatal patterning and anther development processes. Using genetic approaches, Cho et al. presented a possible model where MKK4/MKK5-MPK3/MPK6 are epistatic to INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and HAESA (HAE)/HAESA-like 2 (HSL2) in regulating the programmed separation of cells in the floral abscission zone (Cho et al., 2008). Again, the molecular mechanism of this pathway is poorly understood. Finally, both MKK4 and MKK5 possess a predicted chloroplast uptake sequence in their N-terminus. It was shown that this sequence is crucial for the import of nascent MKK4 polypeptide into chloroplasts in vitro, but it is also required for MKK4 to phosphorylate MPK6 (Samuel et al., 2008).
number of other MAP kinase cascade components possess predicted organelle uptake signal sequences (M. Samuels and B. Ellis, pers. commun.), but these predictions have not yet been tested experimentally.

**Group D MKKs**

Subgroup D MKKs consist of four MKKs, MKK7, MKK8, MKK9, and MKK10. It is unclear whether MKK8 and MKK10 are biologically active because the MKK8 transcripts are not detectable in plants, and MKK10 lacks a properly constructed activation loop target site (Hamel et al., 2006). MKK7 was not characterized using reverse genetic approaches, but rather, was identified as the *bushy and dwarf1* (*bud1*) mutant, in which *MKK7* was over-expressed (Dai et al., 2006). As indicated by the mutant name, MKK7 over-expression caused excessive shoot branching, apparently due to a deficiency in polar auxin transport. In addition, the *bud1* mutant plants accumulated high levels of salicylic acid (SA) and displayed constitutive disease resistance. In contrast, the knockdown of MKK7 expression by use of antisense RNA blocked the induction of systemic acquired resistance, suggesting that MKK7 is a positive regulator of SAR (Zhang et al., 2007b). It will be important to clarify the relationship between auxin polar transport and disease resistance. Recent studies have begun to unveil the linkage between phytohormone imbalance and the outcomes of plant-pathogen interactions (Kazan and Manners, 2009). For example, one report demonstrated that auxin biosynthesis and transport mutants, but not the classical auxin signalling mutant *tir1*, were compromised in SAR establishment (Truman et al., 2010). Beyond the association with auxin-related disease resistance, the substrate(s) of MKK7 remain to be identified. Interestingly, large scale analysis revealed that MPK10 is highly co-expressed with MKK7, and can be phosphorylated by MKK7 on the protein microarray (Menges et al., 2008, Popescu et al., 2009). Therefore, MPK10 could be a candidate for *in vivo* MKK7 target.

The function of MKK9 has been mainly associated with ethylene related physiology. Inducible expression of the constitutively activated form of MKK9 (CA-KK9) leads to increased MPK6 activation, and to elevated ethylene and
camalexin production. The latter two outcomes are compromised in genotypes carrying a \textit{mpk6} knock-out mutation, and partially suppressed by the \textit{mpk3} mutation (Xu \textit{et al}., 2008). Both MPK3 and MPK6 can phosphorylate specific isoforms of 1-aminocyclopropane-1-carboxylic acid synthase (ACSs), which is the rate-limiting enzyme in ethylene biosynthesis (Han \textit{et al}., 2010, Liu and Zhang, 2004), suggesting that a MKK9-MPK3/MPK6 module participates in stress ethylene production. Consistent with these reports, over-expression of MKK9 results in premature leaf senescence, whereas the \textit{mkk9} mutant leaves are more resistant to senescence, a developmental program stimulated by ethylene (Zhou \textit{et al}., 2009). Meanwhile, the MKK9-MPK3/MPK6 module has also been implicated in the ethylene signalling pathway, downstream of ACS and ethylene perception (Yoo and Sheen, 2008). The raf-like MAPKKK CTR1 has long been characterized as an upstream negative regulator of ethylene signalling (Kieber \textit{et al}., 1993). However, the identity of a MAP kinase cassette that could serve as the direct target of CTR1 has not been established. By using the Arabidopsis protoplast system, it was reported that CTR1 activity blocks the action of the putative MKK9-MPK3/MPK6 module in phosphorylating and stabilizing the EIN3 transcription factor, thereby allowing other MPKs to phosphorylate EIN3 at a separate site, which subjects EIN3 to protein degradation (Yoo \textit{et al}., 2008). In contrast to that, a recent study presented evidence that \textit{mkk9} mutant plants do not display ethylene signalling defects, and demonstrated that MKK9 is not required for the ethylene-induced EIN3 stability (An \textit{et al}., 2010). Therefore, how CTR1 affects the phosphorylation and stability of EIN3 is completely obscure. An investigation of MKKs functions in stomatal development identified MKK7 and MKK9 as both negative and positive regulators, depending on when the activated kinases are ectopically expressed in the guard cell lineage (Lampard \textit{et al}., 2009). It requires future study to clarify exactly how they are involved in stomatal development, and what their relationship is with MKK4 and MKK5.
1.3.2 Review of MKK6

1.3.2.1 Cell division and cytokinesis

Cell division, a fundamental process for all living organisms, requires faithful replication of the nuclear genome followed by precise partitioning of the replication products between the daughter cells. Coordination of this complex process involves signalling networks that can integrate environmental and developmental cues to ensure that each division occurs at the correct time and with the appropriate spatial orientation. Cytokinesis forms the final step of the cell division program, and engages elaborately regulated cellular machinery responsible for the physical division of the mother cell cytoplasm containing the replicated genomes.

Somatic cytokinesis in higher plants differs from the process used in animal and yeast cells, where an actomyosin ring contracts centripetally to separate the daughter cells (Balasubramanian et al., 2004). In dividing plant cells, the new cell plate expands centrifugally along a plane marked earlier by a cortical microtubule array, the preprophase band (PPB) (Jürgens, 2005). Golgi-derived vesicles deliver the cell wall and membrane components to the lumen of the expanding cell plate, which ultimately fuses with the parental plasma membrane and cell walls. The delivery of these vesicles is directed by the phragmoplast, a plant-specific structure composed of antiparallel sets of microtubules and actin filaments (Nishihama and Machida, 2001).

In contrast to somatic cytokinesis, the process of meiotic cytokinesis that gives rise to gamete formation is unusual in that neither a preprophase band nor a classical phragmoplast is formed as part of the cell separation process (Otegui and Staehelin, 2000). During microsporogenesis in Arabidopsis, four haploid nuclei are generated within the microspore mother cells, and radial MT arrays are observed originating from each of the nuclear envelopes. These delineate the nuclear cytoplasmic domain (NCD), a zone along which cell division is executed with participation of a special cell plate and a centripetally-closing "mini-phragmoplast" (Otegui and Staehelin, 2004).
1.3.2.1 MKK6 and its orthologues

In 1995, a tobacco MAP kinase, NtNTF6, was cloned and characterized (Wilson et al., 1995), and later shown to be regulated in a cell cycle-dependent manner (Calderini et al., 1998). The kinase activation of NtNTF6 occurs in late anaphase/early telophase, and the NtNTF6 protein is localized at the middle of the separating chromosomes, suggesting that it is probably required for plant cytokinesis. Using the yeast two-hybrid assay, a MPK kinase interacting with NtNTF6 was identified from a tobacco BY-2 cell suspension cDNA library, and designated NtMEK1 (Calderini et al., 2001). NtMEK1 can interact with, and preferentially phosphorylate, NtNTF6 in vitro and in yeast, and its transcript is induced in a cell cycle-regulated manner, similar to that of the NtNTF6, suggesting that NtMEK1 is the upstream MPK kinase acting upon NtNTF6. With the sequencing of the Arabidopsis genome and identification of the Arabidopsis MKK gene family (Ichimura et al., 2002), it became clear that NtMEK1 is likely the tobacco orthologue of MKK6, and that NtNTF6 is the likely orthologue of MPK13. The fact that MKK6 can activate MPK13 when heterologously expressed in yeast, and that the two kinases are co-expressed in different Arabidopsis tissue, suggest that MKK6 and MPK13 might form a functional MAP kinase cascade in Arabidopsis (Melikant et al., 2004).

Meanwhile, the Machida group in Japan initiated a search for interactors of a MAPKKK, NtNPK1, which was first cloned in 1993 (Banno et al., 1993). This group established that NtNPK1 activity increases during cell division, accompanied by a microtubule-associated motor protein, NtNACK1, and that NtNPK1 is essential for the expansion of the phragmoplast during cytokinesis (Ishikawa et al., 2002, Nakashima et al., 1998, Nishihama et al., 2001, Nishihama et al., 2002). By yeast two-hybrid library screening, a tobacco MKK, NtNQK1, and a MPK, NtNRK1, were identified and shown to be acting downstream of NtNPK1 in supporting cytokinesis (Soyano et al., 2003). NtNQK1 turned out to be encoded by the same gene as NtMEK1, while NtNRK1 is identical to NtNTF6. Following these pioneer studies, a microtubule-associated protein (NtMAP65-1) was identified as a target of this MAP kinase cascade, and
its phosphorylation was shown to modulate microtubule dynamics, suggesting that this represented the mechanism by which signalling through NtMEK1/NtNQK1 and NtNTF6/NtNRK1 is affecting the expansion of the phragmoplast (Sasabe et al., 2006). In this manner, a signalling cascade comprised of NtNACK-NtNP-Q-RK-NtMAP65 was characterized as an essential regulator of plant cytokinesis.

Homologues of each of the tobacco NtNACK-NP-Q-RK cascade components can be identified in the Arabidopsis thaliana genome. NtNACK1 has two homologues in Arabidopsis, the AtNACK1/HINKEL kinesin, and AtNACK2/TETRASPORE/STUD. AtNACK1/HINKEL was characterized in two independent studies that showed that it encodes a plant-specific kinesin, and that the gene product was required for the expansion of the phragmoplast before cell-plate formation during cytokinesis (Nishihama et al., 2002, Strompen et al., 2002). TETRASPORE/STUD was identified in a forward genetic screen for male sterile mutants. Multiple mutant alleles of this gene revealed that it is specifically required for meiotic cytokinesis, whereas the vegetative growth is largely unaffected by loss-of-function at the TETRASPORE locus (Hulskamp et al., 1997, Spielman et al., 1997, Yang et al., 2003a). Double mutant studies indicated some level of functional redundancy between these two kinesins (AtNACK1 and AtNACK2), and highlighted their pivotal role in both somatic and meiotic cytokinesis (Oh et al., 2008, Tanaka et al., 2004).

Three Arabidopsis NPK homologues have also been identified and designated ANP1, ANP2, and ANP3 (Krysan et al., 2002). These three genes share unequal redundant function in cell division, with the anp2anp3 double mutant having the strongest growth and cytokinesis defects among the three possible double mutants. In addition, the triple mutant is lethal, suggesting that the ANP kinase family is essential for plant survival. The Arabidopsis NQK1 mutant, anq1, was identified as a severe dwarf whose somatic cells show cytokinesis defects (multi-nucleated cells and incomplete cell walls), suggesting that MKK6 is also involved in somatic cytokinesis (Soyano et al., 2003). However, there is no genetic evidence implicating MPK13 in the cell division process. In short, although loss-
of-function in each of the kinesin, MAPKKK, or MKK gene loci results in cytokinesis defects such as formation of incomplete cell walls and multi-nucleate cells, as well as an overall dwarf growth pattern, there is neither genetic nor biochemical data demonstrating that they indeed form a module and function in a sequential pathway.

Although it seems clear that a MAP kinase cascade involving NtNQK1/NtMEK1 helps regulate cell division, probably by modulating microtubule dynamics, some components of the same proposed cascade have also been found to function in other biological contexts. A study looking for human MEKK1 homologues in plants, found that NtNPK1 was required for N gene-mediated resistance to tobacco mosaic virus (TMV) (Jin et al., 2002). Later, virus-induced gene silencing (VIGS) was used to show that NtNQK1/NtMEK1 and NtNTF6/NtNRK1 were also involved in the same disease resistance pathway (Liu et al., 2004). Taken together, these reports indicate that the NtNP-Q-RK MAP kinase cascade not only regulates cytokinesis, but is also indispensable for disease resistance, at least in some pathosystems.

1.4 Thesis Objective

Although the biological function of NtNQK1/NtMEK1 has been examined in a number of studies, the lack of information about the size and properties of the Nicotiana MPK and MKK gene families makes it uncertain which cascade components might be involved in mediating signals passing through NtNQK1/NtMEK1. On the other hand, the corresponding gene families in Arabidopsis are well delineated, but their biological functions have received scant attention. I therefore hypothesized that the Arabidopsis orthologue of NtNQK1/NtMEK1, MKK6, would also prove to play a role in specific biological processes in this species and that, in doing so, it would interact with discrete upstream activators and downstream targets. The work in this thesis was directed at identifying those processes and players.
CHAPTER 2 MKK6 AND ROOT ARCHITECTURE
2.1 Introduction

The dual-specificity MAP kinase kinases transmit signals by interacting with and phosphorylating MAP kinases on threonine and tyrosine residues of the conserved –TXY- motif. All plant phyla examined seem to possess fewer MKKs than either MAPKKKs or MPKs (Rodriguez et al., 2010), suggesting a common theme that one MKK might be capable of phosphorylating several MPKs. In fact, Arabidopsis makes efficient use of MKKs by recruiting different MPKs to the same MKK in different biological contexts (Andreasson and Ellis, 2010). Among the ten Arabidopsis MPK kinases, MKK6 has been specifically proposed to regulate phragmoplast expansion during cytokinesis, with MPK13 as its downstream module component, based on homology between MPK13 and NtNRK (Soyano et al., 2003). Consistent with such a model, MKK6 was reported to activate MPK13 in yeast (Melikant et al., 2004). MKK6 was also shown to phosphorylate MPK18 when transiently co-expressed with this MPK in Nicotiana benthamiana leaves, but MPK13 was not tested as a substrate in this study (Hua et al., 2006). In a large scale protein microarray screen, MKK6 was found to activate MPK3, MPK6, and MPK8, but again, only 10 MPKs were assayed, and these did not include MPK13 either (Popescu et al., 2009). Thus, while there are indications of several different MKK6-MPK relationships, including the proposed MKK6-MPK13 module, the biological relevance of these heterologous in vitro activities remains unknown, as does the nature of the biological processes in which they might be involved.

The model of an MKK6-MPK13 linkage has a long history from studies of the homologous tobacco module, NtMEK1/NtNQK1-NtNTF6/NtNRK1, the results of which were summarized in the Introduction. NtNTF6/NtNRK1, as well as its alfalfa orthologue, MsMMK3, has been functionally characterized and those functions provide an obvious starting point for understanding the biological role(s) of the Arabidopsis orthologue, MPK13. NtNTF6/NtNRK1 was first identified as a cell cycle-regulated MAP kinase that was localized to the mid-plane of dividing tobacco cells (Calderini et al., 2001, Takahashi et al., 2004). The alfalfa homolog,
MsMMK3, was isolated from a cDNA library on the basis of sequence similarity to NtNTF6 (Bögre et al., 1999). MsMMK3 activity is also high in mitotically-active cells, but, unlike NtNTF6, its activity is microtubule-dependent. The division plane localization of MsMMK3 again supports a role for this kinase in the cytokinesis process. Other studies, however, have suggested that MsMMK3 might be multifunctional. In a comparison of the functions of two alfalfa MKKs, MsPRKK and MsSIMKK, MsMMK3 was shown to be activated by both of them (Cardinale et al., 2002). Interestingly, MsMMK3 was shown to be activated in response to pathogen-derived elicitor but not by salt treatment, suggesting that MsMMK3 might play a specific role in biotic stress response signalling. In addition, MsMMK3 can directly bind to an alfalfa MAPKKK, MsOMTK1 (oxidative stress-activated MAP triple-kinase 1), and to transduce hydrogen peroxide-induced cell death signals (Nakagami et al., 2004).

In contrast to this information concerning NtNTF6/NtNRK1 and MsMMK3, the Arabidopsis homolog, MPK13, is poorly understood. While MPK13 was shown to be co-expressed with MKK6 in roots and buds (Melikant et al., 2004), there are conflicting results concerning the ability of MPK13 to be phosphorylated by MKK6 (Lee et al., 2008, Lin et al., 2010, Melikant et al., 2004, Takahashi et al., 2010). While Melikant et al. (2004) and Lin et al. (2010) found that MPK13 can be activated by MKK6 heterologously in yeast cells and in in vitro kinase assays, respectively. Takahashi et al. (2010) reported that no MPK13 phosphorylation was observed when adding recombinant MKK6, while Lee et al. (2008) observed reduced MPK13 auto-phosphorylation with the addition of MKK6. In addition to these inconclusive phosphorylation results, no phenotype has been described for MPK13 loss-of-function mutants, so a clear picture of the biological function of the proposed MKK6-MPK13 cascade is still lacking.

Genetic tools are powerful in understanding gene functions. Phenotypic analysis of both naturally occurring mutants and genetically modified plants provides important insights into the physiology the gene product regulates. One loss-of-function allele of MKK6 in Arabidopsis, *anq1*, was earlier identified from the Arabidopsis Knockout Facility collection at the University of Wisconsin (Soyano
et al., 2003). The *anq1* mutant is dwarf, and produces multinucleate cells and malformed pollen grains, consistent with a model in which MKK6 is required for successful completion of general cytokinesis. In view of the fact that MPK13 orthologues are involved in cell plate formation, it is reasonable to propose that MKK6 regulates cytokinesis through activating MPK13 (Soyano et al., 2003), but the possibility that it recruits other MPKs in the cell division process cannot be excluded. Beyond the regulation of cytokinesis, it remains unknown whether MKK6 plays a role in Arabidopsis disease resistance responses analogous to the role ascribed to its tobacco orthologue (Liu et al., 2004).

In this chapter, I describe biochemical and genetic approaches I used to study the function of MKK6. Potential MPK targets of MKK6 were identified and the *in vitro* enzyme activity of recombinant MKK6 against these targets was examined. In exploring the biological function of MKK6, I focused on its role in regulating root architecture, and the involvement of MPK13 in this process.
2.2 Materials and Methods

2.2.1 Yeast two-hybrid assay

The yeast two-hybrid system used in this study was the ProQuest™ Two-Hybrid System with Gateway® Technology (Invitrogen). Specifically, MKK6 was first cloned into the pCR8® vector (Invitrogen), and then recombined into the pDEST32 vector by use of the LR reaction. AtMPKs were cloned into the pCR8® or pENTR® vector first, and then transferred into the pDEST22 vector. In this system, pDEST32 is the bait vector and pDEST22 is the prey vector.

Co-transformation was conducted according to the manual. In each co-transformation, 0.5 μg MKK6 in pDEST32 vector and 1.0 μg AtMPK in pDEST22 vector were mixed with the yeast competent cells (MaV203). Co-transformants were selected on the SC-Leu-Trp plates, while positive interactions were selected on the SC-Leu-Trp-His+3-Amino-1,2,4-triazole (3AT) (25 μM) and SC-Leu-Trp-Ura plates. 25 μM of 3AT was added to the SC-Leu-Trp-His plates in order to eliminate the basal HIS3 reporter gene expression generated by protein self-activation.

Quantitative β-galactosidase assays were performed according to the manual (Invitrogen). Red-β-D-galactopyranoside (CPRG) was used as the substrate for β-galactosidase (β-Gal) in this experiment.

2.2.2 Recombinant protein production

The ‘constitutively active’ (CA) MKK6 was generated by QuickChange site-directed mutagenesis (Stratagene) and confirmed by sequencing. Specifically, the conserved S/TXXXXXS/T sites in these MKKs were mutated to DXXXXXE. Native form or mutated genes were cloned into the pGEX 4T-1 or 4T-2 vectors, in which the recombinant proteins were fused with an N-terminal GST tag. Escherichia coli strain BL-21 was transformed with each expression construct and protein production was induced by adding 0.5 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG) to the bacterial cultures at a culture OD600 of 0.4-0.6, followed by incubation at 30°C for 4 hrs. The recombinant GST-fusion proteins
were purified from the cell extract using glutathione-conjugated Sepharose 4B. Protein concentrations were determined with the Bio-Rad protein analysis system using Bovine serum albumin (BSA) as a standard, and the purity of the isolated proteins was assessed by Coomassie Brilliant Blue staining after separation on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels.

2.2.3 In vitro kinase assay

For the *in vitro* kinase assays, 1.0 µg purified GST-MPK was incubated with or without 0.5 µg CA-MKK6 in 25 µl of kinase reaction buffer (50 mM Tris–HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 10 mM MgCl₂, 0.1 mM Na₃VO₄, 0.1 mM ATP, and 5 µCi [γ³²P] ATP) for 30 min at 30°C. For phosphorylation of myelin basic protein (MBP) by MPKs, 1µg MBP was incubated with or without the activated MPKs. SDS-PAGE sample buffer was added to stop the reaction and the reaction mixture was boiled to denature the proteins for SDS-PAGE analysis. Separated proteins in the gel were then transferred to an Immun-Blot™ polyvinylidene fluoride (PVDF) membrane, which was used for autoradiography for an appropriate period of time, followed by Coomassie Brilliant Blue (CBB) staining.

2.2.4 Bimolecular fluorescence complementation (BiFC) assay

A Gateway®-compatible BiFC vector system (pE3136/pE3130) (http://www.bio.purdue.edu/people/faculty/gelvin/nsf/protocols_vectors.htm) was used in this study. pENTRY vectors used in this assay were the same as in the yeast two-hybrid assay. cDNAs of interest were recombined from the pCR®8 vector to the destination vector containing either nYFP (pE3136) or cYFP (pE3130), as indicated. Each vector (10 µg) was transformed into freshly prepared Arabidopsis mesophyll protoplasts prepared according to Wang *et al* (2005). Proteins were allowed to express and interact at room temperature in darkness for 20 hrs following transformation, after which both light field and fluorescence images were taken.
2.2.5 Plant growth condition

For all experiments, Arabidopsis seeds were surface-sterilized, stratified at 4 °C for 2-4 days and germinated on agar-solidified ½ Murashige and Skoog (MS) medium plates (½ strength MS salts, 1% sucrose, 0.5 g/L MES, and 0.7 % agar, pH 5.7). The plates were kept in the growth room at 22-24 °C under 16h/8h photoperiod. One-week-old seedlings were transferred to soil and kept in a growth chamber at 20°C under a 16h/8h photoperiod, unless otherwise indicated.

For root elongation assays, and for cell length and width measurements, images were acquired by using a microscope or direct scanning with a scale reference bar. Measurements were conducted by using the software, ImageJ (http://rsbweb.nih.gov/ij/). Data were analyzed using Excel.

For auxin treatment, five-day-old seedlings were shaken at 50 rpm in liquid ½ MS medium containing in 10 μM NAA for two hours before RNA extraction. For phenotypic analysis, control and RNAi lines were germinated on ½ MS plates before they were transferred on ½ MS medium containing 0.1 μM IAA either with or without dexamethasone (1.0 μM) for another one week before images were taken.

For the pathogen resistance test, 2.5-week-old plants grown in soil were sprayed with *Hyaloperonospora arabidopsidis* Noco2 spore suspension (~5 × 10^4 spores/ml) and then kept in a growth chamber at 80% of humidity and under 16-h-light/8-h-night (Cheng et al., 2009). Spore numbers were then scored 7 days after inoculation by washing off the conidiophores from leaves in water with vigorous vortex, and counting the number using a hemocytometer under microscope.

2.2.6 DNA extraction, RNA extraction and RT-PCR

For DNA extraction, plant tissues were ground in liquid nitrogen and the resulting powder was homogenized in the extraction buffer (0.2 M Tris-HCl, pH 7.5, 0.25 M NaCl, 25 mM EDTA, 1% SDS). After centrifugation at 15,000 rpm for 5 min, DNA was precipitated by adding ½ volume of isopropanol to the supernatant, washed
with 70% ethanol, and resuspended in TE buffer (pH 8.0).

For RNA extraction, plant tissues were ground in powder with liquid nitrogen. Total RNA was then isolated with the RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com/) according to the manufacturer's instructions. Reverse transcription of the RNA (2 μg) was used to synthesize cDNA for each sample using the SuperScript® VILO™ cDNA synthesis kit. (Cat. 11754-050, Invitrogen)

2.2.7 Propidium iodide staining and confocal microscopy

Seedlings were grown vertically on ½ MS plates. Five days after germination (DAG), the seedlings were stained in 1.0 μg/ml propidium iodide for 30 seconds, washed in excess distilled water, and observed under the Nikon Eclipse 80i confocal microscope.

2.2.8 Generating and selecting for Promoter::GUS and RNAi lines

The genomic DNA regions 740bp upstream of the MKK6 gene, and 1534 bp upstream of the MPK13 gene, were used for generation of the corresponding Promoter::GUS lines by Somrudee Sritubtim (PhD thesis, 2005, UBC, Canada). The constructs for these Promoter::GUS lines and RNA interference lines were built and transformed into Arabidopsis thaliana ecotype Columbia (Col-0). I conducted the selection of the homozygous lines from T1 seeds based on antibiotic resistance. T2 seeds were screened on antibiotic-containing plates for selection of lines with one copy of the transgene, based on the segregation ratio (non-resistant vs. resistant seedlings: 1:3). Selected T2 lines were then grown in soil for collecting T3 seeds, from which homozygous lines were selected based on antibiotic resistance or GUS expression.

2.2.9. Histochemical GUS staining

Seedlings of homozygous T3 ProMkk6::GUS and ProMPk13::GUS reporter plants were used for the histochemical GUS staining. One-week-old seedlings grown on ½ MS plates were submerged in 0.5mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining buffer (50mM phosphate buffer, pH 8.0) for 4 hrs, followed by 70% ethanol wash for 3 times until tissues are clear of chlorophyll.
Images were acquired on a Leica DM6000 microscope.

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<th>Primer sequence</th>
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<tr>
<td></td>
<td>Reverse</td>
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<td>5’- CGT TGA TGT ACC GGA ATC TGA C-3’</td>
</tr>
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2.3 Results

2.3.1 MKK6 targets

2.3.1.1 Screening for MKK6 targets – Yeast two-hybrid assay

MKKs are usually considered to be dedicated to the dual phosphorylation and activation of their target MPKs. Since this role would be anticipated to involve specific patterns of protein-protein interaction, I initially looked for downstream target(s) of MKK6 by conducting a directed yeast two-hybrid assay to examine the interactions between MKK6 and each of the 20 Arabidopsis MPKs. Specifically, MKK6 was put into the bait vector, and each of the 20 MPKs in the prey vector of the ProQuest yeast two-hybrid system (Invitrogen). Successful co-transformants of MKK6 and a particular MPK were selected on synthetic complementation growth medium lacking leucine and tryptophan (SC-Leu-Trp). Positive interaction between MKK6 and a MPK was first selected for on the less stringent SC-Leu-Trp-His+3AT (25 µM) medium and then on the more stringent SC-Leu-Trp-Uracil plates.

Among the 20 MPKs tested, I found that MKK6 can interact strongly with MPK4 and MPK11 in the yeast two-hybrid assay, and more weakly with MPK6 and MPK13. This was demonstrated by the fact that co-transformants of MKK6 with MPK4 or MPK11 were able to grow on the most stringent selection medium, SC-Leu-Trp-Uracil plates, whereas the transformants with MPK6, or MPK13 only grew on the less stringent SC-Leu-Trp-His+3AT (25 µM) plates (Fig 2.1 A). This pattern was further confirmed and quantitated using a β-Gal assay (Fig 2.1 B). The strong interaction with both MPK4 and MPK11 is consistent with the fact that MPK4 and MPK11 are paralogous genes. These two kinases belong to group B of the plant MAP kinases, as does MPK13, while MPK6 is in group A. In contrast to MPK4 and 11, two other MPKs in group B, MPK5 and MPK12, did not interact with MKK6, indicating a certain level of specificity in the interactions between MKK6 and its targets (Fig 2.1 B).
**Fig 2.1 Directed yeast two-hybrid screening for MKK6-interacting MPKs.**

A. Dot screening of co-transformants of MKK6 and each of the 20 AtMPKs (indicated by numbers 1-20) on SC-Leu-Trp plate (control), and selective SC-Leu-Trp-His+3AT (25 µM) plate and SC-Leu-Trp-Uracil plate. B. Quantitative β-Galactosidase activity indicating the intensity of protein-protein interactions in the yeast two-hybrid assay. The relative β-Gal units and the standard deviations (SD) for each value are indicated below the graph.
2.3.1.2 Confirmation of protein-protein interactions using the BiFC assay

To validate the Y2H interactions between MKK6 and the four potential target MPKs, Bimolecular Fluorescence Complementation (BiFC) assays were performed using the Arabidopsis mesophyll protoplast system. MKK6 was fused with the N-terminal portion of YFP, and the four MPKs were each fused with the C-terminal portion of YFP. Each pair of vectors was then co-transformed into the protoplast biochemically. Yellow fluorescence can be detected when the two proteins interact with each other and therefore bring the two parts of the YFP in close proximity. The results showed that yellow florescence was detected when MKK6 was co-transformed together with either MPK4, MPK6, MPK11, or MPK13, while co-transformants of n-YFP MKK6 and cYFP-empty vector did not give any detectable fluorescence signal (Fig 2.2). This fluorescence was detected in both the cytosol and nucleus, indicating that the interactions between MKK6 and its target MPKs are not organelle-specific, at least in Arabidopsis mesophyll protoplasts. However, it is still possible that any particular protein-protein interaction may occur in specific cell types and/or within certain subcellular structures during plant development and/or upon stimuli.

**Fig 2.2 Protein-protein interaction assessments by BiFC assays.**

Interaction between MKK6 and MPK4, MPK6, MPK11, MPK13, and empty vector (EV) was assessed by using the BiFC assay. YFP: Yellow Fluorescent Protein signal; BF: Bright field image of the same protoplast.
2.3.1.3 Kinase activities of MKK6 and its MPK targets -- *In vitro* kinase assay

As dual-specificity protein kinases, MKKs phosphorylate their target MPKs on specific threonine and tyrosine residues in the “activation loop” –TXY- motif. To test whether MKK6 can phosphorylate the four putative targets with which it interacts in yeast cells and in Arabidopsis protoplast, *in vitro* kinase assays were conducted, using recombinant forms of the kinases. To this end, GST-fused ‘constitutively activated’ MKK6 (CA-KK6) and the native version of the four MPKs were expressed as recombinant proteins in *E. coli* and purified by using glutathione-Sepharose beads. CA-KK6 and each putative target MPK were then incubated in the kinase assay buffer with $^{32}$P-ATP. Phosphorylated MPK can be visualized by autoradiography after separation in SDS-PAGE gels. When incubated together with CA-KK6, MPK4 phosphorylation was very prominent, while MPK6, MPK11 and MPK13 were more weakly phosphorylated by CA-MKK6 (Fig 2.3 upper panel). It should be noted that MPK6 and MPK13 both showed some capacity for autophosphorylation in the absence of CA-KK6 (Fig 2.3), so that the impact of MKK6 co-incubation had to be assessed as an enhancement above this level of activation.

Despite the known specificity of MKK-catalyzed phosphorylation, evidence of incorporation of radioactive phosphate is not direct evidence for phosphorylation of the activation loop motif. Additionally, autophosphorylation does not necessarily lead to MPK activation. In order to establish whether the phosphorylation of MPKs by MKK6 results in increases in catalytic activity, I used myelin basic protein (MBP) as an artificial substrate for MPKs. Incubation of MBP with or without CA-KK6 give no radioactive signal on the film, indicating that CA-KK6 does not itself phosphorylate MBP. Non-phosphorylated MPK4 has a very low level of activity in phosphorylating MBP (Fig 2.3 lower panel), but when phosphorylated by CA-KK6, this activity was greatly increased. Unphosphorylated MPK11 displayed no detectable MBP phosphorylation activity, but in the presence of CA-KK6, it was able to phosphorylate MBP to a modest extent. Whether the difference in MBP phosphorylation observed between MPK4
and MPK11 reflects the relative affinity of the two kinases for the MBP substrate, or is a function of the extent to which MKK6 can phosphorylate the two MPKs, remains unknown. Native MPK6 and MPK13 were both capable of strongly phosphorylating MBP in the absence of CA-KK6, and the addition of CA-KK6 had little, if any, effect on these activities (Fig 2.3 lower panel).

![Fig 2.3 in vitro kinase assay.](image)

GST-MPK4, MPK6, MPK11, and MPK13 were incubated with or without GST-CA-MKK6 in the kinase buffer. After the reactions were separated on a SDS-PAGE gel, proteins were transferred to an Immun-Blot™ PVDF membrane, which was used for autoradiography (p-MPK and p-MBP), and then CBB staining (MPK and MBP). The asterisk indicates the location of the GST-CA-MKK6 band in the CBB-stained membrane image.
2.3.2 Biological function of MKK6

2.3.2.1 Identification and genetic complementation of mkk6 mutant

It was earlier reported that the loss of MKK6 causes cytokinesis defects in Arabidopsis (Soyano et al., 2003). To further study the biological function of MKK6, another loss-of-function mkk6 mutant (SALK_117230, mkk6-2) was identified from the public mutant collections in the ABRC. In the SALK_117230 line, T-DNA was inserted into the fourth exon of the M KK6 gene (Fig 2.4 A). Homozygous T-DNA insertion mutant plants were selected based on their dwarf phenotype and the predicted genotype was validated by genomic PCR. However, since homozygous mkk6 plants are severely dwarfed and produce few seeds, the mkk6 homozygotes must be obtained from the progeny of selfed heterozygous plants (Fig 2.4 B). The mkk6 mutant plants retained green foliage color when grown on ½ MS plates, but when they were transferred into soil they began to turn dark red, starting from the cotyledons and progressing to other parts of the seedling (Fig 2.4 C). Gradually the whole plant became purple, including the shoots and inflorescences that were developed later. When grown on ½ MS plates for an extended period, the homozygous plants remained green for a longer time, but eventually became chlorotic.

To confirm that the observed severe growth defect phenotype was indeed caused solely by loss-of-function of M KK6, genetic complementation was performed. The native M KK6 CDS driven by the CaMV35S promoter was transformed into heterozygous M KK6+/− plants and the T1 progeny were analyzed. In the T1 generation, three out of ten individual plants were recovered that possessed both the homozygous T-DNA insertion and the transgene, and these plants no longer exhibited dwarfism (Fig 2.4 C & D). This indicates that the severe dwarfism phenotype could be fully rescued by the ectopic over-expression of M KK6, and that the phenotypes observed in the mkk6 mutants were indeed due to the loss-of-function at the M KK6 locus.
Fig 2.4 Identification and characterization of MKK6 T-DNA insertion mutant.

A. T-DNA is inserted into the fourth exon of the MKK6 gene. B. Two-week-old seedlings grown on plates. C. Four-week-old plants grown in soil, showing complementation of the mutant phenotype. D. PCR using Genomic DNA confirms the plant genotype. edMKK6: endogenous MKK6 with introns; exMKK6: exogenous MKK6 without introns.
2.3.2.2 Root cell phenotype of mkk6 mutant

Quantitative measurement of primary root elongation in WT and mkk6 seedlings grown vertically on plates showed not only that the WT primary roots (PRs) elongated faster than those of the mkk6 mutant, but that the root elongation rate increased with time. In contrast, mkk6 PRs continued to elongate at a very slow rate (Fig 2.5 A). Reduced root elongation may be caused by changes in cell division frequency, cell elongation rates, or a combination of these effects. To understand the cellular basis of the retarded root growth in the mkk6 knockout plant, I examined the cell wall integrity in the mutant. I observed that mkk6 mutant plants frequently form incomplete cell walls and cell wall stubs in the root tip (Fig 2.5 B), which is consistent with the published description of the other MKK6 mutant, anq1/mkk6-1 (Soyano et al., 2003).

More detailed examination of the mkk6 mutant root revealed that the root cell morphology is abnormal in different cell types. On average, root cortex cells in mkk6 were only about half the length of those in Col (Fig 2.6 A upper panel & B), suggesting that the root cortex cell anisotropic elongation is defective in mkk6 plants. Interestingly, the radial expansion in the epidermal cells is poorly controlled in the mkk6 mutant, as shown by both the increased cortex cell width and the bulging epidermal cells (Fig 2.6 A & B). In addition, mkk6 mutant plants produce many branched root hairs, which is not a common phenomenon in wildtype root hairs (Fig 2.6 A bottom panel). The twisting observed in the root epidermal cells in the mkk6 mutant (Fig 2.6 A, middle panel) implies that a mechanical force is possibly being created by a differential elongation rate of the cortex cell layer and the outer layer of epidermal cells. Taken together, these data suggest that, in addition to a cell division deficiency, mkk6 mutant plants are also abnormal in directed cell elongation and expansion.
Fig 2.5 Root elongation and cell division phenotype of *mkk6* mutant.

A. Primary root elongation rates in Col-0 and *mkk6* mutant seedlings. B. Propidium iodide staining of five-day-old seedling primary root tip. Yellow arrow heads indicate the incomplete cell walls in the *mkk6* mutant.
Fig 2.6 Root cell morphology of mkk6 mutant.

A. Comparison of WT and mkk6 root cell morphology: Cortex cells (upper panel. Red lines indicate the vector along which the cortex cell length has been measured. Bar=200 µm); epidermal cells (middle panel. Arrow indicates the bulging cell. Bar=400 µm); root hairs (bottom panel. Arrow indicates a branched root hair. Bar=400 µm). B. Quantitative comparison of WT and mkk6 root cortex cell length and width. n=80 and 100 for cell length of Col and mkk6, n=33 for cell width of both genotypes.
MKK6 contributes to maintenance of the root apical meristem structure

The root apical meristem (RAM) is a highly organized tissue locus where cell initials/stem cells divide and then differentiate into various cell types. In WT Arabidopsis plants, four to seven quiescent center (QC) cells in the RAM are located in the middle of the cluster of cell initials, and can be easily distinguished from the other cell types due to their shape and location (Terpstra and Heidstra, 2009). The initials, or stem cell niche, provide a continuous source of derived cells that differentiate into different cell types, such as epidermis, cortex, endodermis, pericycle cells, stele, and columella cells. Since mkk6 plants produce shorter primary roots compared with WT, and root growth is dependent in part on the timely generation of the appropriate initials for the various cell types, I asked whether the RAM is still properly organized in the mutant. When I examined 5-day-old seedlings that had been briefly treated with propidium iodide, which stains the outline of cell walls, I observed that the RAM in wild type root tips was well-organized (Fig 2.7), whereas the mkk6 RAM was disrupted. The QC cells in the mkk6 root tip are either not distinguishable, or have increased numbers (Fig 2.7). I also observed ectopic cell divisions around the QC, which resulted in the surrounding initials becoming disorganized. This ectopic cell division phenotype is different from the cytokinesis deficiency phenotype in other cells, where incomplete cell walls and/or cell wall stubs are often observed.
Fig 2.7 Root apical meristem structure of WT and mkk6 plants.

Propidium iodide staining of 5-day-old WT and mkk6 root tips. Lower panel shows the magnified portions of the upper panel images, with manual drawing of the cell outlines. Stars indicate the QCs.
2.3.3 MKK6-MPK13 cascade

2.3.3.1 Expression patterns of MKK6 and its target MPKs

The observation that MKK6 interacts with several different MPKs in Y2H says and BiFC protoplast assays raises the question of which of these interactions might be biologically relevant, and in which context. In order to examine this question, I first looked for MKK6 co-expressed MPKs. A comprehensive co-transcriptional analysis of 114 Arabidopsis MAP kinases was conducted earlier, using publicly available microarray data (Menges et al., 2008). In this study, MPK4, MPK13 and MPK20 are the MPKs whose expression is most closely correlated with that of MKK6 during plant development, and together they were assigned to the “shoot apex” group. In addition, MKK6 and MPK13 expression patterns are associated with mitotic activity, as are two MAPKKKs, MAPKKK2 and MAPKKK12. In silico analysis using the AtGenExpress Visualization Tool (AVT) supported the idea that MPK13 acts together with MKK6 during plant development, whereas the other three putative MKK6 target MPKs do not share similar expression patterns with MKK6 (Fig 2.8 A). The pattern of co-expression for MKK6 and MPK13 was confirmed experimentally by my own RT-PCR analysis, using RNA from 15 different plant tissues/organs (Fig 2.8 B). MKK6 and MPK13 transcripts could be detected at a relatively low level in young seedlings, and reached their highest levels in the upper part of the stem and mature roots. The RT-PCR analysis also showed that MPK4 and MPK6 are ubiquitously expressed at a relatively high level in most of the plant tissues examined. On the other hand, the MPK4 parologue, MPK11 expression was largely restricted to the upper part of the stem, mature root, and post-mature flowers.
Fig 2.8 Expression patterns of MKK6 and related MPKs.

Comparison of expression patterns of MKK6 and MPK4, MPK6, MPK11, and MPK13 in silico (A) and by RT-PCR (B). (D) after ‘2D hypocotyls’ in panel (B) indicates that the seedlings were germinated and grown in darkness before sample collection. MKK6: At5g56580 (red); MPK13: At1g07880 (green); MPK4: At4g01370 (green); MPK11: At1001560 (blue); MPK6: At2g43790 (lilac). Higher bands in the ‘post-mature flowers’ sample are from the Genomic DNA fragment. C. Histochemical analysis of Promoter\textsubscript{MKK6}::GUS and Promoter\textsubscript{MPK13}::GUS lines.

To obtain a more fine-grained view of these gene expression patterns, I examined Pro\textsubscript{MKK6}::GUS and Pro\textsubscript{MPK13}::GUS transgenic lines which had been generated earlier in the Ellis lab. The result showed that MKK6 promoter activity was concentrated in the root tip and in the sites of incipient lateral root emergence (Fig 2.8 C). In addition, the Pro\textsubscript{MKK6}::GUS activity was only observed within the stele and pericycle cells, but not the cortex or the epidermal cells (Somrudee Sritubtim, PhD thesis, 2005, UBC, Canada). The pattern of MPK13 promoter activity was broadly similar to that of Pro\textsubscript{MKK6}::GUS. Within the root, MPK13 expression, like that of MKK6, was concentrated in the primary root apex and at the sites of lateral root emergence (Fig 2.8 C).
2.3.3.2 MPK13 long and short splicing variants

Because of the high co-expression rate of MKK6 and MPK13, and the pioneering studies of MKK6 and MPK13 orthologues, I studied MPK13 in detail to try to determine whether MPK13 indeed serves as a biological target of MKK6 during development. The genomics databases indicate that the **MPK13** genomic DNA gives rise to two alternative splicing forms, which results in production of a short CDS (coding sequence) variant and a long CDS version of the MPK13 mRNA. A previous published report referred to the short CDS variant as a mis-annotated record (Melikant et al., 2004). However, I was able to clone and confirmed the sequences of both the long and short CDS versions of MPK13 from a total Col-0 cDNA pool, demonstrating that both variants are present in the plant. Using transcript-specific primer sets, I examined the expression of these variants by RT-PCR in different tissues. The results indicated that the expression patterns of the long CDS and the short CDS transcripts are largely similar, with high expression of both forms in mature roots and flowers and buds. However, while the long CDS version was expressed abundantly in the stem, the short version had relatively low expression in that tissue (Fig 2.9 A).

The predicted translation product of the short CDS mRNA would lack 110 amino acids at the C-terminal end, compared with the product of the long variant (Fig 2.9 B). Interestingly, both versions retain the -TEY- activation loop motif (Fig 2.9 B highlighted in yellow), but the short variant lacks the C-terminal acidic site (the common-docking (CD) domain) normally associated with recruitment of other signalling partners such as MKKs, and phosphatases (Fig 2.9 B bold red). To test whether the proposed CD domain is important for the interaction between MKK6 and two MPK13 variants, a yeast two-hybrid assay was carried out, where MKK6 was co-expressed in the yeast cells with either the long or short CDS MPK13 cDNA. As expected, while the long MPK13 protein binds MKK6, the short protein appeared to do so less efficiently (Fig 2.9 C). Since the binding often affects the phosphorylation, I examined whether MKK6-activated MPK13 can phosphorylate MBP. The results showed that while MKK6 can phosphorylate the long MPK13, it does not affect the phosphorylation status of the short MPK13 (Fig 2.9 D).
A

MPK13L

MPK13s

ACTIN

B

K13s
MEKREDGILTYDGRYVMVINLGNIFELSKYIPPIFEPGRAYGICATNSETNEEVAIKKIANAFDN

K13L
MEKREDGILTYDGRYVMVINLGNIFELSKYIPPIFEPGRAYGICATNSETNEEVAIKKIANAFDN

K13s
RVDAKRTLREIKEISSLHMDHDNVMIKIKLDLPEKERFEDVIVYVELMDTLHQLIIRSTQTLTDHCQYFL

K13L
RVDAKRTLREIKEISSLHMDHDNVMIKIKLDLPEKERFEDVIVYVELMDTLHQLIIRSTQTLTDHCQYFL

K13s
YQILRG1KYIHNASNVLRHDRLPNVLNVLNCNKLICDFGLAPTSNETIEITVEVTTPWYRAPHELNLSE

K13L
YQILRG1KYIHNASNVLRHDRLPNVLNVLNCNKLICDFGLAPTSNETIEITVEVTTPWYRAPHELNLSE

K13s
YTGAI DIEWSVGCI FMEILFRETLFFGFSLQYQLKLITEVS-----------------KLP-------

K13L
YTGAI DIEWSVGCI FMEILFRETLFFGFSLQYQLKLITELLG5FD6DLDFLSDNNARKYVQLPKVQKS

K13s
-----------------------

K13L
FREKFPNISSPLMLDAEKMLVFDPSKRITVDEALKQPYLASLHIEINEETPTCPTFP8DFDEETALDEQDIK

K13s
-----------------------

K13L
ELVWRESLMFKNM

C

Sc-Leu-Trp

+  -  -

10μM  25μM  3AT

His

MPK13L

MPK13s

D

-  -  +  -  +  +  CA-KK6

S  S  L  L  -  MPK13

+  +  +  +  +  MBP

p-MPK

← MPK13L

← CA-KK6

← MPK13S

p-MBP

MBP
Fig 2.9 Comparison of the two splicing variants of MPK13.

A. Expression patterns of the long and short CDS MPK13 splicing variants. B. Protein alignment of the long and short CDS variants of MPK13. The -TEY- activation motif is highlighted in yellow and the C-terminal putative CD domain is colored in Red. C. Protein binding affinity of MPK13l and MPK13s with MKK6 in the yeast two-hybrid assay. D. Phosphorylation assay of CA-KK6 on MPK13 long and short proteins, and the activity of MPK13 long and short proteins on MBP as artificial substrate.

2.3.3.3 MPK13 T-DNA insertion allele

In order to learn more about the biological role of MPK13, a T-DNA insertion allele (SALK_130193) was ordered from the ABRC. Interestingly enough, the T-DNA in SALK_130193 is inserted at a location that positions it downstream of the stop codon that defines the short CDS variant, but upstream of the stop codon used in the long variant (Fig 2.10 A). RT-PCR analysis of MPK13 expression in the homozygous SALK_130193 plants confirmed that the insertion disrupts the long CDS transcript, but not the short CDS transcript (Fig 2.10 B). Surprisingly, even though the short MPK13 variant is less efficient in binding with MKK6 and phosphorylating MBP, this allele containing only the short CDS variant displays a WT phenotype throughout its life cycle, and responds to several hormones in an apparently WT manner (data not shown). The ability of homozygous SALK_130193 (mpk13) plants to complete apparently normal growth and development suggests that the weaker yeast two-hybrid association between the short MPK13 protein and MKK6 is not critical to functionality of any signalling cascade in which MPK13 might be engaged, and/or that one or more of the nineteen other Arabidopsis MPKs is able to serve as a redundant partner for MKK6 in the particular biological context(s). The fact that MKK6 can also interact with MPK4, MPK6 and MPK11 in yeast two-hybrid assays would be consistent with the latter suggestion.
Fig 2.10 Identification of SALK_130193.

A. T-DNA is inserted in the fourth exon of the long CDS MPK13 variant but in the 3'UTR of the short CDS variant. Purple bars: exon; blue bars: UTR region; green bars: intron; triangle: T-DNA insertion site in SALK_130193. Numbers below the bars indicate the start site of the exons/introns. B. RT-PCR demonstrated that the SALK_130193 line lost the transcript of the long MPK13 CDS but not of the short CDS.
2.3.3.3 Conditional knockdown allele of *MKK6* and *MPK13*

Since the *MKK6* knockout allele is severely stressed and sterile, and that there is no null knockout allele of *mpk13*, dexamethasone (Dex)-inducible RNA interference lines were generated (Somrudee Sritubtim and Brian Ellis, unpublished data). Multiple independent T3 transgenic lines were selected that showed reduced accumulation of either *MKK6* mRNA or *MPK13* mRNA, respectively, 24 hours after Dex-treatment (Fig 2.11 A). When germinated and grown directly on Dex-containing plates, the transgenic plants showed phenotypes similar to that of *MKK6*, although the severity and uniformity of the phenotype varied in different lines.

When germinated on ½ MS media and then transferred to Dex-containing plates 5 DAG, both the *MKK6 RNAi* and *MPK13 RNAi* genotypes produced far fewer lateral roots (LRs) following Dex induction than did the untreated plants, while Dex treatment of pTA7002 empty vector lines grown in the same manner had no effect on LR formation (Fig 2.11 B & C). Quantitative analysis indicated that the reduction of LR formation was statistically significant (Fig 2.11 C). In contrast, primary root (PR) growth of *MKK6 RNAi* or *MPK13 RNAi* genotypes was largely unaffected by Dex induction in the RNAi suppression lines, indicating that partial suppression of either *MKK6* or *MPK13* results specifically in suppression of LR formation (Fig 2.11 D). In contrast, complete loss-of-function at the MKK6 locus had a significant impact on PR elongation (Fig 2.5), but LR density (expressed as lateral root number per cm of primary root length) in the *mkk6* mutant plants is also significantly reduced (Fig 2.11 E). Thus, even in a genetic context where overall PR architecture is being affected, the ability of those PRs to produce LRs is suppressed. Additionally, the LRs formed in the Dex-treated transgenic plants are mostly located at the basal region of the PRs (Fig 2.11 B). Since these plants were transferred to Dex-containing medium 5 DAG, when the seedlings had already formed lateral root primordia (LRP), this distribution of LRs in the older seedlings suggests that LRP formed earlier ("pre-Dex") can continue to develop into mature lateral roots, whereas Dex treatment, and the associated suppression of *MPK13* or *MKK6* expression blocks the formation of new LRP.
Fig 2.11 Phenotypic analysis of the Dex-inducible *MKK6* RNAi lines.

A. RT-PCR examination of *MKK6* and *MPK13* transcripts in the *MKK6* RNAi lines and the *MPK13* RNAi lines, respectively, 24 hours after Dex-treatment. B. LR reduction phenotype of the RNAi lines. EV: empty vector line. Seedlings were germinated and grown on ½ MS for 5 days before being transferred to ½ MS plates with or without 1 µM dexamethasone. n ≥ 13 C. Quantitative measurement of the LR number in the RNAi transgenic lines. D. Quantitative measurement of the PR length of the RNAi lines. E. Quantitative measurement of the LR and LRP number in the WT and *mkk6* mutant. For all the quantitative measurements, error bars indicate 95% confidence intervals.
2.3.3.4 Phenotypic analysis of the Dex-inducible CA-KK6 lines

Transgenic plants that over-express CA-KK6 driven by a Dex-inducible promoter had been generated earlier in the Ellis lab. The CA-KK6 cDNA is fused with a C-terminal FLAG tag, a small peptide epitope with molecular weight around 8kD. Dex-inducibility of CA-KK6 over-expression was confirmed at the transcript level in two independent transgenic lines (Fig 2.12 A).

Since a reduction in \( MKK6 \) transcript abundance leads to reduced lateral root number, I hypothesized that an increase in \( MKK6 \) transcript abundance would result in production of more lateral roots. After Dex treatment, however, no abnormal root phenotype was observed in 12-day-old CA-KK6 seedlings (Fig 2.12 B), suggesting that MKK6 is required, but not sufficient to induce the lateral root formation in Arabidopsis.

**Fig 2.12 Phenotypic analysis of the Dex-inducible CA-MKK6-Flag lines.**

A. RT-PCR confirmed the over-expression of ectopic \( MKK6-\text{FLAG} \) four hrs after Dex treatment. B. Lateral root number of the transgenic Dex-induced CA-KK6-OX plant lines. Seedlings were grown on \( \frac{1}{2} \) MS plates with or without 1.0 \( \mu \text{M} \) Dex. Lateral root numbers were scored 7 days after germination (DAG).
2.3.3.5 MKK6 and MPK13 and auxin-induced lateral root formation

Auxin is known to inhibit primary root elongation and facilitate lateral root formation (Overvoorde et al., 2010). In order to determine whether a MKK6-MPK13 module might be required for auxin-induced lateral root formation, I first checked if the MPK13 or MKK6 expression is affected by auxin treatment. Even though the MPK13 and MKK6 promoter regions contain putative auxin-responsive elements, both RT-PCR (Fig 2.13 A) and quantitative GUS assay (data not show) showed that their transcript levels were not substantially changed after auxin treatment, which is consistent with the public available microarray data presented by the AtGenExpress Visualization Tool (AVT). In addition, auxin application restored the LR formation deficiency in the Dex-treated RNAi lines (Fig 2.13 B).

Fig 2.13 Auxin, lateral root, and MKK6-MPK13.

A. Auxin responsiveness of MKK6 and MPK13 expression examined by RT-PCR. Five-day-old seedlings were treated by 10 μM NAA or water (control) for 2 hrs before RNA
extraction. B. Auxin restored the LR phenotype of the Dex-inducible RNAi lines. EV: empty vector line. Seeds of different genotypes were germinated on ½ MS plates for three days and then seedlings were transferred on ½ MS medium containing 0.1 μM IAA either with or without Dexamethasone (1.0 μM). Pictures were taken one week after transferring.

2.4 Discussion

2.4.1 Protein-protein interaction and MAP kinase activity

Protein-protein interaction is a common feature of cellular components. By interacting directly or indirectly (connecting through intermediary molecules) proteins can form functional complexes and together, can execute a task. Within the scope of plant MAP kinase signalling, the conserved docking domain of MPK kinases, which consists of a cluster of basic and hydrophobic amino acids (K/R-K/R-K/R-x(1,5)-L-x-L) facilitates the binding of MAPKKs to MAP kinases (Kumar et al., 2008). MKK6 contains a KIKSNLKQLKL sequence near its N-terminus that conforms to this consensus. By using the yeast two-hybrid system, I identified four MPKs, MPK4, MPK6, MPK11, and MPK13, that can interact with MKK6 and these interactions were confirmed by BiFC in protoplast (Fig 2.1 & 2.2). Within the group A MKKs, MKK1 and MKK2 were previously shown to share some common targets with MKK6 in Y2H assays, namely MPK4 and MPK11. MPK6 and MPK13, on the other hand, only interacted with MKK2 and MKK6, but not with MKK1 (Lee et al., 2008). These patterns suggest that a certain level of substrate-specific interaction could form part of the regulation of MKK function.

The interactions between the MKK and MPK are often conditional and transient, and the release of the phosphorylated MPK is reported to be important for their translocalization. For example, the yeast high osmolarity glycerol (HOG) pathway recruits several components of the MAP kinases, many of which display distinct subcellular distributions before and shortly after (<10min) osmotic stress. Hog1, the MAP kinase in this pathway, represent one of the proteins that move from the cytosol to the nucleus upon the treatment (O'Rourke et al., 2002). Similarly, the interactions between MPKs and their targets are also very dynamic, and the disruption of the protein complex upon phosphorylation and/or activation is an
important step in signal transduction. For example, in Arabidopsis, MPK4, MKS1, and WRKY33 form a protein complex. Upon pathogen or pathogenic elicitor challenge, MPK4 is detached from MKS1 and WRKY33, and the latter then binds to, and activates, the promoter of PAD3, an enzyme required for antimicrobial camalexin biosynthesis (Qiu et al., 2008a). Similarly, MPK6 releases ERF104 in response to flg22 and ethylene, allowing the transcription factor bind to its target DNA sequence (Bethke et al., 2009). Another Arabidopsis MAP kinase, MPK3, was reported to phosphorylate VIP1, a transcription factor that is hijacked by Agrobacterium virulence protein VirE2 to facilitate the nuclear import of the Agrobacterium T-DNA (Djamei et al., 2007). These reports suggest the existence in cells of a dynamic and transient protein-protein interaction network that involves both the components within the MAP kinase cascades and the downstream effectors of these signalling modules. The specificity, duration, intensity, and localization of the interactions are thus crucial to the success of the signal transduction.

The specificity of protein-protein interaction is sometimes ensured by a scaffold protein, for which there is experimental evidence in both yeast and mammalian MAP kinase signalling cascades. In plants, however, no such scaffold protein for MAP kinase signalling has been reported yet. However, the fact that the MAPKKK MEKK1, when expressed as a kinase inactive variant, can rescue the mpk4 mutant dwarf phenotype, has led to speculation that MEKK1 may function as a scaffold protein to ensure the correct binding of a particular MKK with MPK4 in disease resistance response pathway (Suarez-Rodriguez et al., 2007).

An experimentally detected physical interaction between a MKK and a MPK does not guarantee that these form a functional phosphorylation module in vivo. NtMEK1, for example, can interact with NtNTF4 in yeast cells more strongly than with NtNTF6. But NtMEK1 preferentially phosphorylates NtNTF6, instead of NtNTF4, in in vitro kinase assays (Calderini et al., 2001). However, which of these results might better portray the in vivo situation is unclear yet. In my study, the intensity of the MKK6-MPK interactions in the Y2H assays was generally correlated with the strength of MKK6 kinase activity when it was tested in vitro.
against recombinant MPKs (Fig 2.3 upper panel). A similar pattern was observed when the changes in phosphorylated MPK activity were assessed by allowing them to phosphorylate a synthetic substrate in vitro (Fig 2.3 lower panel). On the other hand, although MKK6 interacts with both MPK4 and MPK11 to a similar extent in the Y2H assay, it phosphorylates MPK4 much more intensively than it does MPK11. In planta, however, it is possible that interaction with a scaffold protein, or a local accumulation of a particular MKK/MPK pair, could greatly affect these kinase activities, and therefore influence the biological output.

Although interaction is presumed to be pre-requisite for phosphorylation in most studies, apparent exceptions have been documented as well. Arabidopsis MKK3 is able to phosphorylate MPK6, and they together help regulate plant responses to JA (Takahashi et al., 2007). However, this report lacks experimental data demonstrating a direct physical interaction between MKK3 and MPK6, suggesting that the interaction might require other specific proteins and/or occur under specific circumstances. In my experiment, only the four MPKs that showed Y2H interaction with MKK6 were tested as substrates for the MKK6 kinase in activity assays, but the results do not exclude the possibility that other MPKs might be able to serve as downstream targets of MKK6 in vivo. In fact, a screen of ectopically expressed M KK targets in tobacco showed that in the presence of MKK6, MPK18 is phosphorylated (Hua et al., 2006). Interestingly, a recent publication reported the involvement of MPK18 in regulation of cortical microtubule stability (Walia et al., 2009), a phenomenon that could potentially recruit MKK6. Future studies should help determine if MKK6 can phosphorylate MPK18 in vivo and influence MT dynamics.

The canonical MPK model describes MKK-activated MPKs phosphorylating their substrates on proline-directed serine or threonine (-S/TP-) residues, or at a more stringent -PXS/TP- motif. Myelin basic protein (MBP) is commonly used as an efficient artificial substrate for in vitro MAP kinase assays. In some cases, however, recombinant MPKs have been observed to auto-phosphorylate themselves on both Thr and Tyr residues (Seger et al., 1991), and autophosphorylation events on these two sites have been demonstrated to be
intramolecular and independent from each other (Her et al., 1993, Wu et al., 1991). Further studies demonstrated that the monophosphorylated mammalian ERK2/pY and ERK2/pT are catalytically more active than the unphosphorylated ERK2, but less active than the biphosphorylated ERK2/pTpY (Zhou and Zhang, 2002), suggesting that the process of autophosphorylation might modulate signal transduction by interfering with the ability of cognate MKK/phosphatase activities to maintain a specific equilibrium between active and inactive forms of a particular MPK, and/or influencing protein-protein interactions (Pimienta and Pascual, 2007). Autophosphorylation of plant MPKs has been observed in this and previous studies (Lee et al., 2008, Zhou et al., 2009), but the control of autophosphorylation in vitro is not well understood. For example, when two research groups studied the MKK9-MPK6 cascade, Xu et al. (2008) observed negligible MPK6 autophosphorylation, while Zhou et al. (2009) showed that MPK1, MPK3, MPK4, MPK6, and MPK7 all have autophosphorylation activity. Similarly, while Lee et al. (2008) observed MPK4 autophosphorylation, I did not see that in my own kinase assays.

2.4.2 Alternative splicing variants

Alternative splicing (AS) is a general and important mechanism by which eukaryotes regulate gene expression and translation. There are several types of alternative splicing, including exon skipping, exon mutually exclusion, intron retention, and using alternative donor site at the 5’ end or alternative acceptor site at the 3’ end (Reddy, 2007). Through such mechanisms, multiple mRNAs can be derived from a single initial transcript, and in some cases the alternative variants can display different expression patterns, which are controlled spatially and temporally. Some transcriptional variants are subjected to mRNA degradation, which is a way of modulating functional transcript levels. Others generate protein isoforms that differ in protein stability, subcellular localization, enzyme activity, and protein-protein binding affinity (Stamm et al., 2005). In mammalian cells, the three JNK MAP kinases produce ten protein isoforms with different expression patterns, binding abilities, and (presumably) phosphorylation activities (Gupta et al., 1996). Research on plant MAP kinase alternative splicing
is limited but some data are available. Alternative splicing of ANP1 in Arabidopsis generates two protein isoforms, a long protein containing the kinase domain together with an intron-like domain, and a short protein containing only the kinase domain (Nishihama et al., 1997). Yeast complementation experiments indicated that the short protein is more catalytically active than the long protein, suggesting that alternative splicing could be a mechanism by which the cell regulates production of an active ANP1 isoform, but also suggesting that the intron-like domain may contribute a negative regulatory function. The stress-related rice MAP kinase, OsMPK5, is also alternatively spliced to produce a kinase-active isoform and a shorter version without autophosphorylation activity (Xiong and Yang, 2003). Interestingly, three alternative splicing variants of OsBWMK1 were found to localize to different subcellular compartments, as the long and middle length version are both cytosolic and nuclear, while the short version is predominantly localized in the nucleus (Koo et al., 2007). In Arabidopsis, MPK1, MPK2, MPK8, MPK9, MPK11, MPK13, and MPK17 are predicted in the TAIR website to contain transcript variants, but little is known as to the function of these variants. My research showed that the long variant of MPK13 can bind to MKK6 and is catalytically active, whereas the short variant lacks kinase activity (Fig 2.9D). This result largely agrees with a very recent publication, where the authors demonstrated that there are three variants of MPK13, which they designated MPK13 I4, I5, and full length. Even though the MPK13 I4 variant (equivalent to the short version examined in my study) does not have kinase activity, it increases the activity of the full length MPK13 in phosphorylating MBP (Lin et al., 2010). On the other hand, the MPK13 T-DNA insertion mutant provides a unique genetic tool to study the biological function of the long and short MPK13 variants. To my surprise, although the short MPK13 lacks the docking motif and is apparently not catalytically active, the absence of the long version from the T-DNA insertion mutant plants did not cause any abnormal phenotype. It appears that either the short variant is able to function under normal growth condition, or that other MAP kinases can compensate for the loss of the MPK13 long variant in the mutant.
2.4.3 Pleiotropic phenotype of \textit{mkk6} and MKK6 involvement in MT organization

Complete elimination of MKK6 function in the SALK_117230 mutant genotype (\textit{mkk6}) confers massive developmental deficiencies, similar to the phenotype seen in \textit{MKK6 RNAi} seedlings when these are directly germinated and grown on medium containing Dex. The knockout mutant plants are extremely dwarfed and do not produce viable seeds. As with the \textit{anq1/mkk6-1} (WS) mutant, the \textit{mkk6-2} (Col) (which is also \textit{mkk6} in this thesis) mutant also form incomplete cell walls and cell wall stubs. In addition, the progeny of a selfed heterozygous plant segregate at roughly 1:8 ratio of mutant vs. normal seedlings, instead of 1:3, suggesting that MKK6 is required for gametogenesis and/or embryogenesis. These observations support the model proposed by Soyano \textit{et al.} (Soyano \textit{et al.}, 2003) in which MKK6, or its orthologue in other species, plays a central role in proper execution of the plant cytokinesis program. The proposed NtNP-Q-K pathway, in which NtNQK is the tobacco orthologue of MKK6 is conserved in different plant species (Takahashi \textit{et al.}, 2004). On the other hand, since the complete loss of MKK6 is not lethal, \textit{mkk} cells are evidently capable of completing some form of cell division. It is thus possible that MKK6’s role in controlling cytokinesis is not completely essential, or that it does not function in isolation within the cytokinesis program, but shares this role with one or more other MKKs.

In addition to the cell division defects, cellular morphology changes in the \textit{mkk6} mutant suggest a more general role of MKK6 in regulating microtubule (MT) function. Cortical MTs are predominantly plasma membrane-bound MT arrays that appear in specific patterns (Dixit and Cyr, 2004). In elongating root cells, cortical MT arrays are aligned perpendicular to the cell expansion axis (Ehrhardt and Shaw, 2006). Disruption of the transverse alignment of the cortical MTs usually results in cell swelling, such as in the temperature-sensitive \textit{mor1-1} mutant when grown at the restrictive temperature, or in oryzalin treated WT seedlings (Sugimoto \textit{et al.}, 2003), as well as the double MAPKKK mutant, \textit{anp2anp3} (Krysan \textit{et al.}, 2002).
In WT plants, root hairs emerge from the root epidermal cells and elongate as cylindrical, uniaxial structure (Sieberer et al., 2005). Root hair branching can be caused by the reduced expression of α-tubulin in Arabidopsis (Bao et al., 2001), suggesting that the microtubule cytoskeleton is required for the polar tip growth of the root hairs. The expanded cortex cell width, bulging epidermal cells, and branched root hair observed in the mkk6 mutant implies that MKK6 generally regulates the organization of MTs. It is possible that MKK6 and its targets are required for maintaining the cortical MT orientation and/or assembly, and therefore the loss of function of MKK6 leads to the swelling cell phenotype. The proposed NtNP-Q-RK pathway has been shown to target the microtubule-associated protein 65 (NtMAP65-1), whose phosphorylation status affects MT stability and bundling during phragmoplast expansion (Sasabe et al., 2006). Very recently, Beck et al. (2010) reported that anp2anp3 and mpk4 mutants also produce branched root hairs. The authors found that MPK4 physically interacts with MTs and regulates cortical MT bundling through MAP65-1 (Beck et al., 2010). Considering the similar root hair branching phenotype of mkk6, and the fact that recombinant CA-KK6 can phosphorylate MPK4 in vitro, MKK6 is a strong candidate for the missing link between the MAPKKK ANP2/ANP3 and MPK4 in regulating cortical MT bundling.

2.4.4 MKK6-MPK13 helps regulate lateral root formation

Proper root architecture is important to provide the physical anchoring of plants, and to allow them to efficiently absorb water and nutrients (Casimiro et al., 2003). The growing root must constantly integrate environmental and developmental cues to determine both the frequency of lateral root initiation and the location of the lateral root primordia along a parental root (Malamy and Ryan, 2001). Recent studies have made impressive progress in understanding the processes of lateral root initiation, emergence, and elongation.

Lateral roots initiate from a subset of pericycle founder cells positioned opposite the xylem poles (Peret et al., 2009). Through several rounds of organized anticlinal and periclinal cell division, the lateral root initials form dome-shaped
primordia that ultimately break through the endodermis, cortex, and finally the epidermis. The mechanisms that determine which cell becomes the founder cell are not clear. A local auxin concentration oscillation is proposed to trigger previously primed pericycle cells to initiate the cell cycle, and thereby form the initials (Moreno-Risueno et al., 2010). At the same time, mechanical stimuli, such as gravity, can induce additional lateral root formation at the outer side of the root bending site (Ditengou et al., 2008). Both models are based on cellular responses being induced by a local auxin maximum. Therefore, auxin seems to be the main force for the triggering re-entry of a cell into the cell cycle and thus initiating formation of a new lateral root.

MKK6 and MPK13 are co-expressed developmentally, and Promoter\textsubscript{MKK6}::GUS and Promoter\textsubscript{MPK13}::GUS activities are observed in stele and pericycle cells, the subset of which form the lateral root founder cells. Concentrated GUS activity driven by these promoters is observed at lateral root primordia and in the primary root tip, where auxin accumulates. However, MKK6 and MPK13 GUS activity is more diffuse than the pattern of DR5::GUS activity, which is a commonly used sensor for local auxin concentrations, and there is no evidence that MKK6 and MPK13 transcript accumulation is directly regulated by auxin. Instead, the MKK6-MPK13 cascade could be acting as a largely pre-existing signalling module in those tissues that are responding to the local auxin maximum and other developmental signals. Once activated by those signals, the MKK6-MPK13 module could facilitate cell division by phosphorylation of cell cycle-regulated proteins. In fact, MPK13 orthologues in tobacco and alfalfa are both activated in a cell cycle-regulated manner and localized to the newly formed cell plate during cytokinesis (Bögre et al., 1999, Calderini et al., 2001).

Partial suppression of either MKK6 or MPK13 leads to reduced numbers of lateral roots, indicating that both genes are required for LR formation. Interestingly, while the knockdown of MKK6 and MPK13 by growth on 1\mu M Dex at post-germination developmental stages seems to block the cell divisions that initiate or facilitate lateral root formation, this signalling deficiency does not interfere with active cell division in the primary root tip. This suggests a dosage-
dependent regulatory machinery, reminiscent of the concentration-dependent effects of auxin in modulating primary root and lateral root growth. For example, exogenous application of auxin at a concentration of $10^{-7}$ M clearly inhibits primary root growth, whereas the same concentration of auxin promotes lateral root branching (Fig 2.14) (Nacry et al., 2005, Rahman et al., 2007). Similarly, a ‘transcript dosage’ of MKK6-MPK13 that is able to fully sustain primary root tip cell division, may be insufficient to facilitate the lateral root initial cell division.

Since MKK6 can also interact with and phosphorylate three other MPKs, namely, MPK4, MPK6, and MPK11, it was important to establish whether these MPKs are also involved in regulating lateral root formation. mpk4 plants are dwarf and contain incomplete cell walls in leaf epidermal tissue, as well as branched root hairs (Beck et al., 2010, Petersen et al., 2000). However, lateral root formation in mpk4 (Ler) plants is comparable to that of WT plants (Su et al., 2007). Intriguingly, while mpk6-2 was shown to produce short primary roots and more adventitious roots at the hypocotyl/root interphase, lateral root formation is not affected (Müller et al., 2010), another more recent report presented normal mpk6-2 lateral root developmental phenotype compared with WT (Wang et al., 2010b). Whether this discrepancy is a reflection of different growth media used in different labs awaits clarification. Finally, mpk11 mutant plants do not display any distinguishable phenotype under the conditions that have been examined (Chapter 3). Collectively, these data suggest that a MKK6-MPK13 cascade specifically regulates lateral root formation in Arabidopsis, while MPK4, MPK6, and MPK11 could be tightly controlled biological targets of MKK6 in other developmental pathways.

Despite the numerous research reports linking MKK6 and MPK13, the evidence for phosphorylation of MPK13 by MKK6 is neither conclusive nor consistent (Lee et al., 2008, Lin et al., 2010, Melikant et al., 2004, Takahashi et al., 2010). My own kinase assays showed that MPK13 can be activated weakly by MKK6, and the phosphorylated MPK13 showed modestly increased activity in phosphorylating MBP, results that support a weak, but positive regulation of MPK13 by MKK6.
Taken together, my results suggest that the MKK6-MPK13 module is required for initiating and/or sustaining pericycle cell division during lateral root initiation. The observation that partial suppression of either \textit{MKK6} or \textit{MPK13} gene expression resulted in formation of far fewer lateral roots, without compromising primary root growth, provides further evidence for this developmental specificity.

2.4.5 MKK6 contributes to root apical meristem maintenance

In recent years, the influence of hormone homeostasis and crosstalk on meristem organization has become an important research focus in plant biology (Terpstra and Heidstra, 2009). Among these phytohormones, auxin concentration maxima and gradients generated by local auxin biosynthesis and polar transport are believed to have a major influence on root apex pattern formation (Grieneisen \textit{et al.}, 2007, Petersson \textit{et al.}, 2009). These and other studies have clearly demonstrated the presence of an auxin concentration maximum in the root tip quiescent center (QC) cells, a mitotically inactive cell cluster that is required for organizing and maintaining the surrounding stem cells. During QC cell establishment, cytokinin transiently antagonizes auxin and plays a negative regulatory role in specifying the root stem cell niche (Müller and Sheen, 2008). Recently, ABA has also been shown to help maintain the QC quiescence and inhibit stem cell differentiation (Zhang \textit{et al.}, 2010a). Additionally, in a genetic screen for deregulated QC cell division, ethylene (ET) overexpression mutants were identified, demonstrating that ethylene induces the division of QC cells during postembryonic development of the root apex (Ortega-Martinez \textit{et al.}, 2007). The loss-of-function mutant of the MAPKKK CTR1, which has increased endogenous ET levels and extremely short primary roots (Kieber \textit{et al.}, 1993), was shown to undergo ectopic QC cell division. Interestingly, MPK6 can phosphorylate and stabilize the ethylene biosynthesis enzymes ACS2 and ACS6, thereby leading to increased ET levels (Liu and Zhang, 2004). Since MPK6 can interact with MKK6, and its phosphorylation is increased when co-incubated with CA-KK6, I decided to examine ET-related gene expression patterns in the \textit{mkk6} mutant. Interestingly, I was able to show that several ET biosynthesis genes and ET-responsive genes are up-regulated in \textit{mkk6} seedlings (Fig 2.14 A), raising the
possibility that loss of MKK6 function interferes with local ET homeostasis, and thereby affects root meristem organization. However, neither the ethylene signalling inhibitor AgNO3 nor the ET biosynthesis inhibitor, AOA, could rescue the mkk6 mutant phenotype (Fig 2.14 B & C), suggesting that the ectopic cell division observed in the mkk6 mutant root tip is not directly caused by elevated ET levels, and/or that MKK6 is epistatic to ET in controlling root stem cell division.

In addition to hormone homeostasis, organelle machinery such as vesicle trafficking systems, also contribute to the organization of stem cell niche. Müllar et al. (2010) described the short root and disorganized root apical meristem phenotype in the mpk6 mutant, which is reminiscent of the mkk6 mutant root tip. The authors showed that MPK6 localizes to distinct spots in the preprophase band (PPB) and phragmoplast of dividing cells, and to the plasma membrane (PM), and trans-Golgi network (TGN) vesicles of non-dividing cells (Müller et al., 2010). This subcellular localization of MPK6 suggests a possible role in cell plate formation, which might explain the ectopic cell division observed in the mpk6 mutant root meristem. However, how the MPK6-related vesicle system contributes to the cell division plate/orientation is unclear yet. Nevertheless, in this regard, MKK6 could be the upstream MKK of MPK6 in facilitating the movement of vesicles, which might be associated with MT function and with regulating cell division in root apical meristem maintenance.

Further research is needed to help us understand whether the MPK6 stimulation of ET biosynthesis and the MPK6 mediated vesicle trafficking are related to each other in the context of cell division and differentiation, and how MKK6 is involved in preservation of the root apical meristem.
Fig 2.14 Ethylene and MKK6.

A. Quantitative RT-PCR of ethylene biosynthesis genes and responsive genes expression in mkk6 mutant. Effect of ethylene biosynthesis inhibitor (AOA) or ethylene signalling inhibitors (Ag⁺) on primary root elongation (B) and Lateral root density (C) of Col and mkk6. Seedlings were grown on ½MS plates for five days before being transferred to plates containing either AOA or Ag⁺. Primary root length and lateral root numbers were measured one week after transferring. In B & C, lighter bars represent WT, whereas darker bars represent mkk6. Error bars indicate 95% confidence intervals.
2.4.6 MKK6 in disease resistance

The tobacco orthologue of MKK6, NtMEK1/NtNQK1, is required for the N-mediated disease resistance (Liu et al., 2004). As shown above and in other studies, MKK6 can also interact with and phosphorylate MPK4 and MPK6, both of which are also responsive to pathogen infection (Bartels et al., 2009, Beckers et al., 2009, Gao et al., 2008, Han et al., 2010, Meszaros et al., 2006, Petersen et al., 2000, Qiu et al., 2008a, Qiu et al., 2008b, Suarez-Rodriguez et al., 2007, Zhang et al., 2007a), and mkk6 displays dwarf phenotype similar to that of mpk4. These data led me to ask whether MKK6 itself might play a role in disease resistance. To this end, I first challenged mkk6 mutant plants with the virulent oomycete pathogen, *Hyaloperonospora arabidopsidis* (*Noco2*). When maintained under the same growth conditions, mkk6 plants supported an equal amount of oomycete growth on their leaves compared with WT (Fig 2.15 A). The pathogenesis-related genes, PR1 and PR2, which are highly expressed in the mpk4 mutant, and serve as an indication of constitutively activated disease resistance and elevated salicylic acid accumulation (Petersen et al., 2000) showed no increase in expression in mkk6 plants beyond what was observed in WT plants. In mkk6 mutant, however, either of the genes display increased expression compare with WT (Fig 2.15 B). The reduced PR1 expression in mkk6 mutant is not reproducible (data not shown). It has also been reported that the dwarfism of mutants such as mekk1 and mpk4 that are resistant to pathogen infection can often be rescued by growing the plants under higher temperature or higher humidity conditions, (Su et al., 2007, Suarez-Rodriguez et al., 2007), although the mechanism of this rescue is not understood. However, when grown at elevated temperature (29°C), mkk6 plants showed even greater retarded growth instead of a recovered phenotype (Fig 2.15 C). Collectively, these data do not provide support for the idea that MKK6 is involved in plant disease resistance, at least not in R gene-mediated disease resistance. However, given the wide spectrum of plant pathogens and associated elicitors, it is impossible to conclude that MKK6 is absolutely not required for any disease resistance.
Fig 2.15 Disease resistance phenotype of \textit{mkk6}.

A. Pathogen infection phenotype of WT and \textit{mkk6} plants. 2.5-week-old soil-grown plants were sprayed with \textit{H. a.} Noco2 spore suspension. The number of conidia was scored one week after treatment. B. \textit{PR1} and \textit{PR2} expression in WT and \textit{mkk6} plants. C. WT and \textit{mkk6} seedlings growing under normal and elevated temperature conditions.
CHAPTER 3 MPK4 IS REQUIRED FOR MALE-SPECIFIC MEIOTIC CYTOKINESIS IN ARABIDOPSIS
3.1 Introduction

The life cycle of flowering plants consists of the vegetative phase and the reproductive phase. Plant reproduction requires successful formation of both female and male gametophytes that are embedded within the female reproductive organ, the ovule, and the male reproductive organ, the stamen. The stamen is composed of filaments and anthers, and the male gametes develop within the anther sac (Sanders et al., 1999). Unlike animals, which establish their germ lines early during embryogenesis, plants initiate their gametophyte late in the reproductive phase from somatic tissues (Boavida et al., 2005). This process is a programmed event with highly organized cell expansion, cell division and cell differentiation to ensure proper patterning of specialized tissues/cells that are required for the success of sexual reproduction.

Following the commitment to a male reproductive cell lineage, specific sporophytic cells undergo meiosis, a special cell division that marks the transition from the diploid microspore mother cells (MMCs) to the haploid microspores, involving both sporophytic and gametophytic genes (Caryl et al., 2003, Ma, 2005). In Arabidopsis, the MMCs undergo two synchronized rounds of meiosis to produce tetrads that are clustered and surrounded by a callose wall. The callose wall is then degraded by callase, releasing the microspores from each other (Scott et al., 2004). Individual microspores continue receiving signals, nutrients, and cell wall components from both the sporophytic tapetum and their endogenous gametophytic gene products until these microspores become mature pollen grains and are released from the anther locules. The whole meiosis process is complicated and includes many aspects, such as chromosome rearrangement and segregation, cell cycle regulation, cell proliferation and differentiation, cell polarity control, as well as programmed cell death (Caryl et al., 2003, Ma, 2005).

Mitogen-activated protein (MAP) kinase cascades are conserved signalling modules found in all eukaryotic cells (Ichimura et al., 2002). In Arabidopsis, several MAP kinases have been reported to regulate a variety of developmental
processes, including plant reproduction. MPK3 and MPK6 are two paralogous genes that regulate both female and male gamete formation. *mpk6* null mutants are partially sterile under certain growth conditions due to the reduced size of their anthers, but not due to the inviability of the pollen (Bush and Krysan, 2007). In *mpk6*/ *mpk3* mutant plants, the cell division of ovule integuments is arrested at late stages, resulting in female sterility (Wang *et al*., 2008a). The same mutant plants are also defective in cell differentiation during anther development, and thus produce less viable pollen than WT (Hord *et al*., 2008). In addition, loss of function in the plasma membrane-localized MAPKKKs, MAP3Ke1 and MAP3Ke2A, results in deficiencies after pollen mitosis I that affect pollen viability (Chaiwongsar *et al*., 2006). These two MAPKKKs work as gametophytic genes, as the mutant pollen grains are non-viable, and homozygous plants could not be isolated.

MPK4 is one of the most extensively studied Arabidopsis MAP kinases and is the only MAP kinase to be identified by a forward genetic screen. It is known that MPK4 is post-translationally activated by various environmental stresses, such as cold, drought, touch, wounding, and salt (Ichimura *et al*., 2000). However, when the *mpk4* mutant was identified, it appeared to behave similarly to wildtype plants in response to these stresses (Petersen *et al*., 2000). Instead, the *mpk4* phenotype indicated that MPK4 participated in plant responses to biotic stress, and that it negatively regulated SA-dependent systemic acquired resistance (SAR), while positively regulating the JA pathway through EDS1 and PAD4 (Brodersen *et al*., 2006). The severe dwarfism of *mpk4* is only partially rescued by ectopic expression of *nahG* (Petersen *et al*., 2000), which suggests that the dwarf phenotype is partly caused by elevated SA level, but also argues for other possible functions of MPK4 that may cause dwarfism when absent. Upstream of MPK4, MEKK1 (MAPKKK) and MKK1/MKK2 (MKKs) are required for the flg22-induced activation of MPK4, where MKK1 and MKK2 apparently function in a redundant manner (Gao *et al*., 2008, Huang *et al*., 2000, Meszaros *et al*., 2006, Qiu *et al*., 2008b, Suarez-Rodriguez *et al*., 2007). Interestingly, Rodriguez *et al*. (2007) showed that the structure, but not the kinase activity of MEKK1, was
required for the activation of MPK4 in the context of disease response, implying that MEKK1 may act as a scaffold for this particular MAP kinase module configuration, rather than as a catalytically active MAPKKK. Knockout alleles of the components in the same cascade impair ROS signalling as well (Pitzschke et al., 2009). Upon activation by flagellin treatment, MPK4 binds and phosphorylates its substrate, MKS1 (MPK4 substrate1) (Andreasson et al., 2005, Caspersen et al., 2007). MKS1 interacts with both MPK4 and the transcription factor, WRKY33, in the absence of flg22 treatment, but this protein complex collapses upon perception of flg22, releasing WRKY33 to bind to and activate the promoter of PAD3, an enzyme required for camalexin production (Qiu et al., 2008a). As noted above, MPK4 can also be activated by wounding, low temperature, and hyper-osmolarity, which suggests it has role in abiotic stress responses (Droillard et al., 2004, Ichimura et al., 2006, Ichimura et al., 2000). However, the role MPK4 plays in the abiotic stress signalling network remains unknown.

Although mpk4 plants were reported to produce fewer pollen grains compared to WT (Petersen et al., 2000), the mechanism of this apparent partial male sterility is unknown. In this chapter, I have examined the fertility of the mpk4 mutant. In mpk4 mutant plants, anthers can develop normal microspore mother cells (MMCs) and peripheral supporting tissues, but the MMCs fail to form an intersporal callose wall after male meiosis, and thus cannot complete meiotic cytokinesis. Nevertheless, the multinucleate mpk4 microspores subsequently proceed through mitotic cytokinesis, resulting in enlarged mature pollen grains that possess increased sets of the tricellular structure. This pollen development phenotype is reminiscent of those observed in both atnack2/tes/stud and anq1/mkk6-1 mutants. Protein-protein interaction analysis enabled me to define a putative signalling module linking AtNACK2/TES/STUD, ANP3, MKK6 and MPK4. In addition, I found that activated MPK4 can phosphorylate a pollen-specific microtubule-associated protein, MAP65-9. Taken together, these results suggest that an AtNACK2-ANP3-MKK6-MPK4 cascade specifically facilitates male meiotic cytokinesis in Arabidopsis.
3.2 Materials and Methods

3.2.1 Molecular cloning and expression pattern analysis

MPK4 was cloned into the pENTR vector and MPK11 was cloned into the pCR8 vector, both of which are Gateway™ Entry vectors.

Plant tissues were collected and frozen in liquid nitrogen. After being ground, total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN). Total RNA (2 μg) was used for synthesizing cDNA from each sample using Superscript II (Invitrogen). PCR was conducted using JumpStart (Sigma) and the gene-specific primers listed in Table 3.1.

3.2.2 Generation of Pro<sub>MPK4</sub>::MPK11 in mpk4 transgenic lines and genotyping of mpk4/mpk11 double mutant

The promoter region of MPK4 was PCR-amplified and inserted into pUC19 using SalI and Ncol digestion sites. The MPK11 CDS was amplified and inserted into the same vector, using Ncol and ClaI digestion sites. The whole Pro<sub>MPK4</sub>::MPK11 fragment was then restriction enzyme-digested and ligated into a similarly digested pZP211 binary vector, which was then transformed into mpk4 mutant plants by floral dip. T1 generation plants were selected by germinating seeds from the dipped plants on plates containing both kanamycin (for the mpk4 mutation) and gentamycin (for the transgene). In the T2 generation, mpk4 homozygous plants were then selected, based on their dwarf phenotype. The primers used for amplifying the appropriate DNA fragments are shown in Table 3.1.

3.2.3 Plant growth conditions and seedling treatment

For all experiments, Arabidopsis seeds were surface-sterilized, stratified at 4 °C for 2-4 days and germinated on agar-solidified ½ Murashige and Skoog (MS) medium plates (½ MS salt, 1% sucrose, 0.5 g/L MES, and 0.7 % agar, pH 5.7). The plates were kept in the growth room at 22-24 °C under 16h/8h light-dark cycle. One-week-old seedlings were transferred to soil and kept in a growth
chamber at 20°C under a 16h/8h light-dark cycle.

For salicylic acid treatment, 4-week-old Col plants growing in soil were sprayed with a 250 μM SA solution. For ABA treatment, one-week-old Col seedlings growing on ½ MS plates were picked out and shaken in liquid ½ MS media with or without 10 μM ABA solution on a rotary shaker at 50 rpm. Leaves or whole seedlings were harvested by freezing in liquid nitrogen at the indicated time points before RNA extraction.

3.2.4 RNA extraction and gene cloning

Total RNA from different plant tissues was isolated with the RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com/) according to the manufacturer's instructions. Reverse transcription was performed using a first-strand cDNA synthesis kit (Invitrogen). Genes of interest were amplified from the cDNA and cloned into the pCR®8/GW/TOPO® vector (Invitrogen). The sequences of the primers used for the cloning are shown in Table 3.1.

The MPKs and MKKs of interest had been cloned into the pCR8 vector earlier (Lee et al., 2008).

3.2.5 Generation of Promoter::GUS lines and histochemical GUS assay

A DNA fragment representing the 740bp region immediately upstream of the M KK 6 coding region was cloned into the pCAMBIA1381Z vector (Somrudee Sritubtim, PhD thesis, 2005, UBC, Canada). DNA fragments of 908bp upstream of the M PK 4 coding region, and 569bp upstream of the M PK 11 coding region, were each cloned into a modified pUC19 vector, and then sub-cloned into the pZP211 binary vector (Wang et al., 2008b) to generate the corresponding Promoter::GUS lines. The sequences of the primers used to amplify the promoter regions are shown in Table 3.1. These particular promoter sequences were selected because they represent the entire region between the gene of interest and the 3’-neighbour gene.

Histochemical staining for GUS activity was performed by submerging the harvested inflorescence in a solution of 0.5 mg/mL X-Gluc (5-bromo-4-chloro-3-
indolyl-\textit{beta}-D-glucuronic acid, cyclohexylammonium salt) in sodium phosphate buffer (50 mM, pH 7.0, and 0.1% Triton® X-100) for 4 hrs, followed by clearing in 75% ethanol. Images were acquired on a dissecting microscope with mounted camera.

\textbf{3.2.6 Staining and microscopy}

Pollen viability staining was performed according to Alexander (Alexander, 1969) and the images were acquired on a Leica DM6000 microscope.

For Toluidine Blue staining, samples were fixed in FAA (formalin (3.7% formaldehyde)-acetic (5% glacial acetic acid)-alcohol (50% ethanol)) overnight and then embedded in Spurr’s resin (Spurr, 1969). Semi-thick transverse sections (0.5 µm) of the developing anthers were obtained on an Ultracut instrument (Leica EM UC6) and stained with 0.05% Toluidine Blue in 0.1 M phosphate buffer at pH 6.8 (O’Brien \textit{et al.}, 1964).

Scanning electron microscopic images were acquired on a Hitachi S-2600N Variable Pressure Scanning Electron Microscope.

For nuclear staining, fresh pollen grains were collected by shaking the opened flowers in 0.1 M sodium phosphate buffer pH 7.0, collecting the grains by centrifugation, and treating these with DAPI staining buffer (0.1 M sodium phosphate, pH 7.0, 1 mM EDTA, 0.1% Triton X-100, 1.0 µg/mL 4’-6-diamidino-2-phenylindole (DAPI)) (Park \textit{et al.}, 1998).

For Aniline Blue staining, flower tissues were fixed in 10% acetic acid in ethanol for 1.5 hrs and softened by submerging in 1.0 M NaOH overnight. After washing in 50 mM KPO\textsubscript{4} buffer (pH7.5), pollen grains were stained with 0.01% Aniline Blue in the same KPO\textsubscript{4} buffer (Besser \textit{et al.}, 2006) and observed using a confocal microscope under UV light.

For transmission electron microscopy, \textit{WT} and mutant flower buds were dissected and anthers were fixed by high pressure freezing (Kaneda \textit{et al.}, 2008). Samples were then freeze-substituted in 2% osmium tetroxide and 8% dimethoxypropane in acetone from -80°C to -20°C for 4 days, followed by an
overnight substitution at 4°C. The temperature was gradually raised to room temperature over 2 hrs and samples were infiltrated with a gradient series of Spurr’s resin to a final concentration of 100% resin. The infiltrated samples were then polymerized in fresh Spurr’s resin at 60°C. Blocks containing anthers were cut to 50nm thick sections on a Reichert Ultracut E microtome. Slide-mounted sections were stained with uranyl acetate for 6 min and then with lead citrate for 12 min. Images were then acquired on a Hitachi H7600 transmission electron microscope.

3.2.7 Recombinant protein production and \textit{in vitro} kinase assay

Recombinant proteins were cloned into the pGEX 4T-1 or 4T-2 vectors, which express the recombinant proteins with an N-terminal GST tag. Procedures for cloning, transformation, protein expression and purification are as described in the Materials and Methods section in Chapter 2. The following primers were used for cloning MAP65-9 into the pGEX 4T-1 vector: forward, 5' - CCGGAATTCCATGTCCAAATCTCAAATCGAATCAAC-3'; reverse, 5' - CCGCTCGAGTCAGCCATGGCGTGATAGAG-3'.

The \textit{in vitro} kinase assay was also performed according to the Materials and Methods section in Chapter 2. Specifically, 1µg purified MAP65-9 was added to the reaction mixture together with 5 µCi [γ-32P] ATP for 30min, and the indicated MPKs, activated or not activated by CAKK6.

3.2.8 Yeast two-hybrid assay

Yeast two-hybrid assays were conducted with the ProQuest™ yeast two-hybrid system (Invitrogen). cDNAs of interest were recombined from the pCR®8 entry vector to either pDEST32 (pDB) or pDEST22 (pAD) vectors, as indicated. Selected pairs of vectors were introduced into the yeast strain MaV203, and co-transformants were selected on synthetic complete dropout medium without leucine and tryptophan (SC-LT). Positive interactions were selected on the less stringent SC-LT medium lacking histidine but including 25µM 3-amino-1,2,4-triazole (3AT) (SC-LT-His+3AT) as well as on the more stringent SC-LT medium lacking uracil (SC-LT-Uracil).
### 3.2.9 Bimolecular fluorescence complementation (BiFC) assay

A Gateway®-compatible BiFC vector system was used in this study (http://www.bio.purdue.edu/people/faculty/gelvin/nsf/protocols_vectors.htm). cDNAs of interest were recombined from the pCR®8 vector to the destination vector containing either nYFP (pE3136) or cYFP (pE3130), as indicated. Each vector (10 µg) was transformed into freshly prepared Arabidopsis mesophyll protoplasts as described in Wang et al (2005). Proteins were allowed to express and interact at room temperature in darkness for 20 hrs following transformation, after which both light field and fluorescence images were acquired.

### 3.2.10 In vitro pollen germination

Fresh pollen grains were collected from WT and mpk4 mutant plants, respectively, by gently tapping open flowers above the in vitro pollen germination plates (5 mM MES (pH 5.8 adjusted with TRIS), 1.0 mM KCl, 10 mM CaCl₂, 0.8 mM MgSO₄, 1.5 mM boric acid, 1% (w/v) agar, 16.6% (w/v) sucrose, 3.65% (w/v) sorbitol, and 10 µg ml⁻¹ myo-inositol (Fan et al., 2001)). Plates loaded with pollen were put in the growth chamber at 22-24 °C for 16 hours, when germinated pollen grains were imaged using a Leica DM6000 microscope.

#### Table 3.1 Primers used for the work in Chapter 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fw/Rv</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPK4</td>
<td>Forward</td>
<td>5'- gcgCATATGTCGGCGGAGAGTTTCTTTC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'- gcgATCGATTCACACTGAGTCTTGAGG-3'</td>
</tr>
<tr>
<td>MPK11</td>
<td>Forward</td>
<td>5'- gcgCCATGGGcgATGCAATAGAAACC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'- gcgATCGATTTAAGGTTAACTTGACTG-3'</td>
</tr>
<tr>
<td>MPK11s</td>
<td>Reverse</td>
<td>5'- TCAATGTTTTTTCCAAATTGAAACG-3'</td>
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<tr>
<td></td>
<td>Forward</td>
<td>5'- gcgATCGACTCAATCGGTGCTAAGCTA-3'</td>
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<tr>
<td>ProMK4</td>
<td>Reverse</td>
<td>5'- gcgCCATGGGCGAGCAAATTTCCAC-3'</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>5'-ccggaattcGCTCTCTCTCTCTCTCTTCAGCGAG-3'</td>
</tr>
<tr>
<td>ProMKK6</td>
<td>Reverse</td>
<td>5'-cgccaatcatcTTTTTTTTTTTTTTTTTTTCTGCTAGAGG-3'</td>
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<td>Forward</td>
<td>5'-CGGCTGGTACGACTGAGCAGAGGATTGC-3'</td>
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<tr>
<td>ProMPK11</td>
<td>Reverse</td>
<td>5'-CGGCTGGTACGACTGAGCAGAGGATTGC-3'</td>
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</table>
### Table 3.1 Primers used for the work in Chapter 3

<table>
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<tr>
<th>Gene</th>
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<td>Tes-C</td>
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<td>Reverse 5'- CTAGAGATGCAACAAAGTTGGATATG-3'</td>
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<tr>
<td>ANP3</td>
<td>Forward 5'- ATGCAGGATATTTCGGATCGG-3'</td>
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</tr>
<tr>
<td></td>
<td>Reverse 5'- CTATCCTTTTGGCCTGATAATGG-3'</td>
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<td>MAP65-9</td>
<td>Forward 5'- ATGTCGAAATCTCAAATCGAATCAAC-3'</td>
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<tr>
<td></td>
<td>Reverse 5'- TCAGCCATGCGTCGTAGGAGG-3'</td>
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<td>GSL1</td>
<td>Forward 5'- GACGAGGTGAGATGGAGTAACAC-3'</td>
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<td>Reverse 5'- GACGAGGTGAGATGGAGTAACAC-3'</td>
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<td>Forward 5'- GGTACCATTTCCTCTTCTTCG-3'</td>
<td></td>
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<tr>
<td></td>
<td>Reverse 5'- GGTACCATTTCCTCTTCTTCG-3'</td>
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<tr>
<td></td>
<td>Reverse 5'- AAATCCAGAAAGGCTGCTTGTC-3'</td>
<td></td>
</tr>
<tr>
<td>GSL5</td>
<td>Forward 5'- CTGAAGCTCTGCAACACTCG-3'</td>
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<tr>
<td></td>
<td>Reverse 5'- GAATCGCTCTGAGTTGAGGAACCTCC-3'</td>
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<tr>
<td>GSL6</td>
<td>Forward 5'- ACTGTTGGTCTGCTCGAGAGG-3'</td>
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<tr>
<td></td>
<td>Reverse 5'- AGGCAGTAAATGCTGGTCTTC-3'</td>
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</tr>
<tr>
<td>GSL8</td>
<td>Forward 5'- GTCAAGGTCATGAGCGTGTAG-3'</td>
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<td></td>
</tr>
<tr>
<td>GSL9</td>
<td>Forward 5'- TCGTGTAGCCCAAAGACGTAGAG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse 5'- TACTATGAGATGCGAGGGAAC-3'</td>
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</tr>
<tr>
<td>GSL10</td>
<td>Forward 5'- ATTTCTAGAGCTGGCGATGAG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse 5'- AGTTGAGTTGCGATTTTGTCGT-3'</td>
<td></td>
</tr>
<tr>
<td>ACTIN8</td>
<td>Forward 5'- ATTAAAGCTCGTGGCA-3'</td>
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</tr>
<tr>
<td></td>
<td>Reverse 5'- TCCGAGTTGAGAGGCTAC-3'</td>
<td></td>
</tr>
<tr>
<td>GUS</td>
<td>Forward 5'- ATGTTACGTCTCTGAGAAACCCCAACC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse 5'- AGACTTCCCGCTGATACCAGACGGTTC-3'</td>
<td></td>
</tr>
</tbody>
</table>
3.3 Results

3.3.1 Study of the paralogous gene pair, MPK4 and MPK11

3.3.1.1 Cloning and characterization of MPK11 long and short variants, and comparison between MPK4 and the long MPK11 variant

MPK4 is most closely related to MPK11, which belongs to group B of the family of plant MAP kinases, and represents one of the uncharacterized MAP kinases. The Arabidopsis gene annotation for MPK11 indicates that two splicing variants are derived from the same gene. The long CDS is 1100bp, while the other variant retains the fourth intron, which introduces a pre-stop codon and produces a short CDS with 828bp. This pattern is similar to the MPK13 splicing variants described earlier (pp. 45-48). I first examined whether both MPK11 transcripts exist in the plant. RT-PCR results showed that both the long and short transcripts of MPK11 could be detected (Fig 3.1 A). However, while the long version is visualized with 30 cycles of PCR amplification after separation on agarose gel, the short version gave only faint bands after 36 cycles of PCR, demonstrating that the short transcript abundance is much lower than that of the long transcript. Nevertheless, the successful cloning of the MPK11 cDNA (long variant) rules out the possibility that MPK11 is a pseudogene, and my analysis confirms that both long and short transcripts are present in Arabidopsis.

The gene structure of the long MPK11 is very similar to that of the MPK4 (Fig 3.1 B), and the encoded protein shares 88.3% identity with MPK4 at the amino acid sequence level. Such a high degree of similarity implies that these two proteins may have similar overall structure and a similar ability to interact with other proteins. To test this idea, I co-expressed MPK4 and MPK11 in yeast with each of the ten MKKs, looking for the interacting MKKs in a yeast two-hybrid assay. These assays showed that both MPK4 and MPK11 can interact with MKK1, MKK2, and MKK6, with the strongest interactions seen between MKK2 and MPK4/MPK11 (Fig 3.1 C), consistent with the observation of Lee et al. (2008). The same MKK interaction profiles of MPK4 and MPK11 suggested that they might be functionally interchangeable on other levels, as well.
Fig 3.1 Comparison between MPK4 and MPK11.

A. RT-PCR amplification of the long and short splicing variants of \( MPK11 \) transcripts from different Arabidopsis tissues. 1. Post-mature flowers; 2. Upper stems; 3. Rosette leaves; 4. Roots. \( gMPK11s \): \( MPK11 \) short variant from the genomic DNA. B. Schematic drawing of gene structures of \( MPK4 \) and \( MPK11 \). Purple bar: exon; blue bars: UTR.
region; green bars: intron. Numbers below the bars indicates the length (in bp) of the exons/introns/UTRs. C. AtMKKs interaction profiles of MPK4 and MPK11. Table indicates the two constructs that are co-expressed in the yeast colonies displayed in each panel above. Upper panels show the colonies obtained with AtMKKs in the bait vector, and MPK4/MPK11 in the prey vector; lower panels are colonies with MPK4/MPK11 in the bait vector, and AtMKKs in the prey vector

3.3.1.2 Expression patterns of MPK4 and MPK11

To gain knowledge about MPK11 functionality, I examined the expression patterns of the long MPK11 variant using different approaches, and compared these with the expression pattern of MPK4. Both publicly available microarray data and my semi-quantitative RT-PCR analysis with RNA samples from 15 different plant tissues/organs at various developmental stages, showed that MPK4 mRNA is present ubiquitously and abundantly everywhere throughout the development process (Fig 2.8 A & B). On the contrary, differences in MPK11 transcript abundance are striking, with relatively high levels in post-mature flowers, in the upper part of the stem, and in mature roots, and very low levels elsewhere (Fig 2.8 A & B). These data indicated that MPK4 and MPK11 transcripts are differentially controlled by their promoters, both spatially and temporally.

To learn more about the tissue-specific expression of MPK4 and MPK11, I generated ProMPK4::GUS and ProMPK11::GUS transgenic lines. The GUS activities driven by the promoters of MPK4 and MPK11 generally agree with the RT-PCR results that MPK4 is more abundantly expressed than MPK11. In addition, MPK4 promoter activity is concentrated at the shoot apex and lateral root tips (Fig 3.2 i & ii), regions where active cell division occurs. In flower organs, GUS activity driven by the MPK4 promoter is observed most strongly in pollen grains, while MPK11 promoter activity is seen in the parental tissue of the anthers, but not in the pollen grains (Fig 3.2 v & v').
Fig 3.2 Histochemical analysis of $Pro_{MPK4}::GUS$ and $Pro_{MPK11}::GUS$ lines.

GUS activity in transgenic Arabidopsis plants at different developmental stages. i and i': 10 DAG seedlings; ii, iii and ii', iii': 3-week-old seedlings; iv, iv' and v, v': 6-week-old blooming plant flowers. Lower panels present the enlarged images of v and v'.
The Genevestigator database predicts that the transcript of MPK11 is dynamically regulated by various stimuli, such as cold, salt, ozone, etc. In addition, MPK11 expression might be associated with senescence, because ethylene can promote its expression, and MPK11 transcripts are highly abundant in post-mature flowers, where the tissues are undergoing senescence. Transcription of MPK11 has also been reported to increase after treatment with the stress hormone, ABA (Xin et al., 2005), and I was able to confirm through semi-quantitative RT-PCR analysis that MPK11 expression is up-regulated by treatment with ABA and SA (Fig 3.3 A & B). The ABA-induced MPK11 expression is quite transient, while SA-induced expression is more sustainable. Interestingly, MPK11 transcript levels are also elevated in mutants that constitutively accumulate high levels of SA (Fig 3.3 C) (Li et al., 2001). These data indicate that the promoter activity of MPK11 is inducible, rather than constitutive, and that MPK11 may therefore be a stress-induced gene whose function is restricted to certain conditions.

![Fig 3.3 MPK11 transcription is induced by SA and ABA.](image)

RT-PCR analysis of MPK11 expression is induced by SA (A) and ABA (B). MPK11 mRNA abundance is increased in mutants accumulating high levels of endogenous SA (C).
3.3.1.3 *MPK11* loss-of-function mutant

One available *MPK11* T-DNA insertion mutant (SALK_049352) was identified from the SALK Institute mutant collection. In this mutant, the T-DNA was inserted in the third exon of the *MPK11* gene (Fig 3.4 A), and therefore disrupts both the long variant and the short variant. Homozygous SALK_049352 plants were isolated by screening progeny using genomic PCR, and loss of the full length *MPK11* transcript in these homozygous plants was confirmed by RT-PCR (Fig 3.4 B). Unlike the *mpk4* mutant, the *mpk11* mutant’s vegetative growth is indistinguishable from that of the WT, and it did not display any fertility defects under normal growth conditions.

![Fig 3.4 Identification of *mpk11* mutant.](image)

**Fig 3.4 Identification of *mpk11* mutant.**

A. Genomic organization of the MPK11 gene and the T-DNA insertion position within the SALK_049352 line. Arrows indicate the relative place of the primers used for Amplification of MPK11 from Col wildtype and from homozygous plants by RT-PCR. B. Absence of full length MPK11 in homozygous SALK_049352 examined by RT-PCR.

Then I looked for conditional phenotype in the *mpk11* mutant. Various hormone treatments were applied to this mutant, together with WT controls, and the resulting phenotypes were examined (Table 3.2).

For a biotic stress response, the *mpk11* mutant was challenged with the oomycete pathogen, *H. a. Noco2*, which develops abundant white spores on WT leaves one week after inoculation. The *mpk11* mutant supported the growth of
this oomycete in a manner similar to WT.

Since MPK11 is shown to be up-regulated by ozone treatment (predicted by Genevestigator) in the collective public microarrays, three-week-old mpk11 and WT plants were treated with 500ppm ozone for up to 24 hours. I found that mpk11 is neither markedly more resistant to ozone treatment nor more sensitive to the stress, since it shows leave curling symptoms at the same time as does WT.

Table 3.2 List of treatments and phenotypic traits examined for mpk11

<table>
<thead>
<tr>
<th>treatment</th>
<th>condition</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noco2</td>
<td>2.5-week-old plants were sprayed with P. parasitica Noco2 spores (~3 × 10⁴/ml)</td>
<td>Spore growth examined one week after infection</td>
</tr>
<tr>
<td>NAA</td>
<td>0.1 and 1.0 µM NAA plates</td>
<td>Primary root length and lateral root number</td>
</tr>
<tr>
<td>6-BA</td>
<td>1.0 µM 6-BA plates</td>
<td>Seedling growth</td>
</tr>
<tr>
<td>BL</td>
<td>1.0 µM BL plates</td>
<td>Seedling growth</td>
</tr>
<tr>
<td>ABA</td>
<td>1.0 µM ABA plates</td>
<td>Germination rate</td>
</tr>
<tr>
<td>ACC</td>
<td>10 µM ACC plates</td>
<td>Hypocotyl length of two-day-old seedlings in dark</td>
</tr>
<tr>
<td>GA</td>
<td>1.0 µM GA plates</td>
<td>Germination rate</td>
</tr>
<tr>
<td>Ozone</td>
<td>500 ppm for 24 hrs</td>
<td>Three-week-old plants were tested and leaf curling was observed</td>
</tr>
<tr>
<td>NaCl</td>
<td>0, 50, 100, 200 mM NaCl</td>
<td>Seedling growth on plates</td>
</tr>
<tr>
<td>Cold</td>
<td>4°C</td>
<td>One-week-old seedling were put at 4°C for 24 hrs followed by normal growth</td>
</tr>
<tr>
<td>Water loss</td>
<td>Measurement of detached leaf weight over 5 hrs</td>
<td>Leaves from four-week-old plants</td>
</tr>
</tbody>
</table>
MKK2 has been shown to mediate cold and salt stress signalling (Teige et al., 2004), and the public microarray data suggests that the transcription of MPK11 is regulated by both cold and salt. Since MKK2 and MPK11 can interact with each other, it seemed possible that MPK11 might also mediate cold and salt stress, by acting downstream of MKK2. To test this, I examined whether mpk11 knockout plants also share a similar phenotype as mkk2. Col and mpk11 plants were grown on ½ MS plates with 0mM, 50mM, 100mM or 200mM NaCl. Similarly, 24-hour cold treatment (4°C) did not cause phenotypic difference between WT and mpk11 either. However, no visible difference in terms of germination and seedling size was observed up to 10 days of growth under these conditions.

Taking these findings together, the lack of phenotypic difference between the mpk11 mutant and WT plants upon different treatments leaves open the question as what the biological role of MPK11 is. Moreover, it is likely that the restricted expression pattern of MPK11 makes it impossible to compensate for the loss of function of MPK4 in the mpk4 mutant.

3.3.1.4 Promoter swap of MPK4 to MPK11

Since the mpk4 mutant is severely dwarfed, whereas mpk11 has no visible phenotype, and since MPK11 expression pattern is relatively restricted, I hypothesized that MPK11 cannot functionally compensate for the loss of MPK4 in mpk4 plants due to this restricted expression. Given the high sequence similarity of the two proteins, and the same profile of interactions with upstream MKKs, I asked whether MPK11 could serve as the functional equivalent to MPK4 if it were expressed under the control of the MPK4 promoter. To test this hypothesis, I designed a ‘promoter swap’ experiment, in which the MPK11 CDS was fused with the MPK4 promoter, and the resulting construct was transformed into mpk4 mutant plants.

In the original mpk4 mutant, aDs element that encodes a kanamycin-resistant (Kan+) gene and a GUS gene was inserted into the first intron of the MPK4 gene (Petersen et al., 2000, Sundaresan et al., 1995), and I therefore cloned the ProMPK4::MPK11 fragment into a pZP211 binary vector, which confers gentamycin
resistance (Genta⁺) to the transgenic plants. After *Agrobacterium*-mediated transformation of *MPK4/mkp4* heterozygous plants, T1 seeds were collected and the transgenic seedlings were selected on plates containing Carb+Kan+Genta antibiotics. After selfing and setting seed, T2 seeds were collected individually from 10 WT-looking plants. Progeny from an individual T1 (double heterozygous plant) are expected to segregate according to the Mendelian inheritance rule (Table 3.3). To select single transgene lines, collected seeds were screened by sowing on Kan⁺ or Genta⁺ plates, and the segregation ratios of different antibiotic resistances were scored (Table 3.3 and 3.4). Four lines that displayed unexpected segregation ratios were removed from the collection (Table 3.4 red ratios). Seeds from the other six lines were selected on plates containing both Kan⁺ and Genta⁺. Green seedlings, whose genotypes are in green in table 3.3, were transferred into soil, and these seedlings displayed either WT or mpk4 (dwarf) phenotype. If normal segregation takes place, and if Pro$_{MPK4::MPK11}$ does not compensate for the loss of *MPK4*, the ratio of the plants displaying WT or mpk4 phenotypes should be 2:1 (Table 3.3). The actual numbers of plants displaying these different phenotypes are presented in Table 3.5. The discrepancies between the expected numbers and the observed numbers are likely due to the small sample size.

<table>
<thead>
<tr>
<th>AB</th>
<th>Ab</th>
<th>aB</th>
<th>ab</th>
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<tbody>
<tr>
<td>AB</td>
<td>AABB</td>
<td>AABb</td>
<td>AaBB</td>
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<td>Ab</td>
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<tr>
<td>Ab</td>
<td>AaBb</td>
<td>AaBb</td>
<td>aaBb</td>
</tr>
</tbody>
</table>

The presence of Pro$_{MPK4::MPK11}$ (A) confers seedlings Gentamycin resistance (red), whereas the presence of *mpk4* (b) confers kanamycin resistance (blue). Genotypes that are resistant to both antibiotics are shown in green. Within the green genotypes, dwarf plants resembling *mpk4* are highlighted in yellow, whereas plants resembling WT phenotype are not. Seedlings of aaBB genotype are not resistant to any antibiotics.
Table 3.4 Segregation ratio of Pro\textsuperscript{MPK4::MPK11}/mpk4 T2 seedlings

<table>
<thead>
<tr>
<th># Line</th>
<th>\textbf{Kan}\textsuperscript{+} (20 μg/ml) Exp 1:3*</th>
<th>\textbf{Genta}\textsuperscript{+} (50 μg/ml) Exp 1:3</th>
<th>\textbf{Kan}\textsuperscript{+} Genta\textsuperscript{+} Exp 7:9 (0.78)</th>
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<tbody>
<tr>
<td>L1</td>
<td>15:30</td>
<td>10:44</td>
<td>26:58 (0.45)</td>
</tr>
<tr>
<td>L2</td>
<td>12:27</td>
<td>11:43</td>
<td>42:65 (0.65)</td>
</tr>
<tr>
<td>L3</td>
<td>10:36</td>
<td>4:50**</td>
<td>-</td>
</tr>
<tr>
<td>L4</td>
<td>10:33</td>
<td>17:37</td>
<td>55:53 (1.04)</td>
</tr>
<tr>
<td>L5</td>
<td>16:33</td>
<td>18:36</td>
<td>52:56 (0.93)</td>
</tr>
<tr>
<td>L6</td>
<td>19:33</td>
<td>15:39</td>
<td>43:65 (0.66)</td>
</tr>
<tr>
<td>L7</td>
<td>5:44</td>
<td>14:40</td>
<td>-</td>
</tr>
<tr>
<td>L8</td>
<td>7:40</td>
<td>11:43</td>
<td>49:59 (0.83)</td>
</tr>
<tr>
<td>L9</td>
<td>16:35</td>
<td>7:47</td>
<td>-</td>
</tr>
<tr>
<td>L10</td>
<td>17:41</td>
<td>3:51</td>
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</table>

\* Ratios are non-resistant seedlings vs. resistant seedlings

\** Red ratios indicate that the lines likely contain more than one insertion and were therefore omitted from the following experiments.

Table 3.5 Number of normal/dwarf plants (T2) resistant to both Kan and Genta in six independent lines

<table>
<thead>
<tr>
<th># Line</th>
<th>normal</th>
<th>dwarf</th>
<th>Normal/dwarf Ratio (exp 2:1)</th>
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<tr>
<td>L1</td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>L2</td>
<td>6</td>
<td>5</td>
<td>1.2</td>
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<tr>
<td>L4</td>
<td>6</td>
<td>4</td>
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</tr>
<tr>
<td>L6</td>
<td>3</td>
<td>9</td>
<td>0.33</td>
</tr>
<tr>
<td>L8</td>
<td>5</td>
<td>7</td>
<td>0.71</td>
</tr>
</tbody>
</table>
If $Pro_{MPK4}::MPK11$ can rescue the $mpk4$ phenotype, I predicted that some WT-looking plants should contain no copy of the wildtype $MPK4$ gene. To test this, I randomly selected three normal plants and one dwarf plant from each of the eight lines, and used genomic PCR to check whether they contain wildtype $MPK4$. The results showed that all the normal appearing plants contained $MPK4$, while none of the dwarf plants contained $MPK4$, although they do contain $Pro_{MPK4}::MPK11$ (Fig 3.5 A). Interestingly, in one line I was not able to detect a $Pro_{MPK4}::MPK11$ insert, even though that line had been selected for Genta resistance.

To make sure that $MPK11$ was indeed being expressed from the $Pro_{MPK4}::MPK11$ transgene in the $mpk4$ mutant, I examined $MPK11$ expression levels in 20 individual dwarf plants, by RT-PCR. The whole Inflorescence with cauline leaves, flowers and buds was used to extract RNA. Fig 3.5 B shows that $MPK11$ is indeed highly expressed in several of the selected plants (indicated by red stars).

In theory, if the $Pro_{MPK4}::MPK11$ construct cannot rescue the $mpk4$ phenotype, the T2 plants should display a ratio of 2:1 of normal to dwarf plants. But if the construct can rescue the $mpk4$ phenotype, the ratio should be higher than 2:1. Although the ratios reported in Table 3.5 are not exactly 2:1, none of them is higher than 2:1, supporting that the construct is not able to rescue the $mpk4$ dwarfism. The result that $MPK11$ expression, driven by the $MPK4$ promoter, cannot compensate for the loss of function of $MPK4$, strongly suggests that either $MPK11$ lost its function, or that it is neofunctionalized in a manner that it can no longer complement key $MPK4$ functions. In any case, $MPK11$ is not functionally equivalent to $MPK4$ in planta.
Fig 3.5 Confirmation of Pro\textsubscript{MPK4}::MPK11 expression in the mpk4 mutant background.

A. Genotyping of the transgene (Pro\textsubscript{MPK4}::MPK11) and endogenous MPK4 in normal (1, 2, 3) and dwarf (4) plants by genomic PCR. B. Confirmation of elevated MPK11 expression driven by the MPK4 promoter in individual dwarf plants.
3.3.1.5 Generation of mpk4/mpk11 double mutant

To test whether MPK4 and MPK11 are genetically redundant, I crossed mpk4 and mpk11 plants to generate the double mutant. If MPK11 does not maintain any function, then the double mutant would be predicted to phenocopy mpk4. However, if MPK11 is neofunctionalized but has retained some functional redundancy with MPK4, the double mutant should be more severe than mpk4 or lethal.

For genotyping, genomic PCR was used to confirm the loss of MPK11, whereas loss of MPK4 was scored by its dwarf phenotype. Out of 179 individual progeny from the cross, no mpk4/mpk11 double mutant was identified (Table 3.6), suggesting that the double mutant might be embryo lethal. However, not only did I find no mpk4/mpk11 double mutant, the ratio for either the single mpk4 mutant or single mpk11 mutant genotype was lower than expected (Table 3.6). Therefore, I checked the exact genotype of 80 individual plants, which are progeny resulting from selfing of a single double heterozygous plant (MPK4+/− MPK11+/−), aiming to find the reason for the apparent discrepancy. Given that MPK4 is located on the fourth chromosome, and MPK11 on the first, the segregation of the two genes should follow the Mendelian inheritance rule for a dihybrid cross, as shown in Table 3.7. However, the experimental results are different from the predicted values. The number of MPK4+/−MPK11+/− progeny is higher than expected, so are the numbers of MPK4+/−MPK11+/− and MPK4+/− MPK11+/+ progeny. This pattern does not agree with the Mendelian inheritance law of independent assortment. Rather, it suggests a chromosomal linkage between these two genes which will be discussed in 3.4.2.
Table 3.6 Identification of \textit{mpk4/mpk11} double mutant

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<tr>
<td>In theory (total 179)</td>
<td>100.7</td>
<td>33.56</td>
<td>33.56</td>
<td>11.2</td>
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<tr>
<td>Results</td>
<td>140</td>
<td>16</td>
<td>23</td>
<td>0</td>
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Table 3.7 Genotypes of the progeny obtained from a self-pollinated individual \textit{MPK4^{+/-}MPK11^{+/-}} plant

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expected ratio</th>
<th>Expected (out of 80)</th>
<th>Results</th>
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<tbody>
<tr>
<td>\textit{MPK4^{+/-}MPK11^{+/-}}</td>
<td>1/16</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>\textit{MPK4^{+/-}MPK11^{+/-}}</td>
<td>1/8</td>
<td>10</td>
<td>14</td>
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<td>1/8</td>
<td>10</td>
<td>19</td>
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<td>\textit{MPK4^{+/-}MPK11^{+/-}}</td>
<td>1/4</td>
<td>20</td>
<td>37</td>
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<td>\textit{mpk4^{+/-}MPK11^{+/-}}</td>
<td>1/16</td>
<td>5</td>
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<td>\textit{mpk4^{+/-}mpk11^{+/-}}</td>
<td>1/16</td>
<td>5</td>
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3.3.2 MPK4 is required for male meiotic cytokinesis

3.3.2.1 *mpk4* plants are pollen-defective but *mpk11* plants are not

MKK6 has been previously shown to be a positive regulator of cytokinesis (Soyano *et al.*, 2003), and proposed to act through MPK13. I demonstrated in the previous chapter that MKK6 can interact with MPK6, MPK13, MPK4, and MPK11, which is consistent with a recent report (Lee *et al.*, 2008).

MPK4 and MPK11 form a paralogous set of kinases that share 88.3% amino acid sequence similarity. However, while loss of function at the MPK4 locus is known to have severe phenotypic consequences (Petersen *et al.*, 2000), I found that the homozygous *mpk11* knock-out mutant (SALK_049352, Fig 3.4) is indistinguishable from wildtype (*WT*). To explore this apparent functional distinction, I made reciprocal crosses between *mpk4* and *mpk11* plants, and found that crosses using *mpk11* pollen on *mpk4* stigmas generated normal seed set (Fig 3.6, left panel), whereas siliques resulting from pollination with *mpk4* pollen on *mpk11* stigmas contained no seeds (data not shown). Siliques originating from *mpk4* self-pollinated flowers were short and contained only a small number of seeds. To exclude the possibility that the observed pattern is caused by a genetic interaction between the two mutant loci, I conducted reciprocal crosses between *mpk4* and *WT* Ler plants. This showed that *WT* pollen grains could fertilize *mpk4* mutant flowers, but that *mpk4* pollen grains could not fertilize *WT* flowers (Fig 3.6, right panel). These observations suggested that the *mpk4* mutant has specific functional defects in the male, but not in the female, reproductive organs.

While *mpk4* floral organs are generally smaller than *WT* (Fig 3.7 A), the overall floral morphology of *mpk4* mutants resembles *WT*, indicating that the observed male infertility could not be attributed to gross structural deficiencies. Alexander staining of the protoplasm of mature pollen grains (Alexander, 1969) suggested that the *mpk4* mutant pollen grains are largely nonviable (Fig 3.7 B). Scanning electron microscopic examination of the morphology of *mpk4* stamens and pollen demonstrated that *mpk4* anthers contained far fewer pollen grains compared to
the WT anthers (Fig 3.7 C). In addition, while WT pollen grains were oval and symmetrical, with typical midline germination apertures, the mpk4 mutant pollen grains were generally much larger and round, rather than oval, and frequently possessed distorted external morphology, including an abnormal germination aperture.

**Fig 3.6 mpk4 has reduced male fertility.**

Crosses between mpk4 (female) and mpk11 (male) (left panel) and reciprocal crosses between mpk4 and Ler (right panel). Siliques resulting from crosses are indicated by a red arrow, siliques from mpk4 self-pollination by blue. k4 is mpk4 in the right panel. Scale bar = 1 cm
**Fig 3.7 Morphological phenotype of *mpk4* anther and pollen grains.**

A. Ler WT and *mpk4* intact flowers. B. Alexander staining of Ler WT and *mpk4* mature pollen grains. Scale bar = 10 µm. C. Scanning electron microscopic views of Ler WT and *mpk4* anther and pollen grains. Scale bar = 100 µm in the upper panel, 10 µm in the lower panel.
3.3.2.2 Developing *mpk4* pollen cannot undergo normal male meiotic cytokinesis

To further examine the impact of loss of *MPK4* function on the process of microsporogenesis, I sectioned *mpk4* anthers at different developmental stages. At stage 5, the *WT* anther primordium forms four locules, and the microspore mother cells (MMCs) can typically be visualized within the locules (Fig 3.8, (Sanders *et al.*, 1999)). The MMCs enter meiosis at stage 6, followed by meiotic cytokinesis at stage 7, at which point the resulting haploid microspores are bounded by a thick callose wall to form tetrads (Fig 3.8). This callose wall is enzymatically degraded by callase at development stage 8, which allows the microspores to be released from the tetrads. Individual microspores then become polarized, and continue two rounds of mitosis to form the mature pollen grain.

In the *mpk4* mutant, anthers were found to develop normally to form locules and MMCs at stage 5 (Fig 3.8), but as the MMCs finish meiosis, no callose wall appears between the microspores. At later developmental stages, most of the *mpk4* microspores are enlarged and contain multiple nuclei (Fig 3.8 and Fig 3.10). Empty locules, or locules containing apparently aborted pollen grains, are also observed in the *mpk4* mutant anthers at later stages of flower development.

To further confirm the absence of callose wall formation during microspore development in the *mpk4* mutant, I stained whole *mpk4* flower buds with Aniline Blue, which binds to callose and emits fluorescence under ultra-violet light (Enns *et al.*, 2005, Spielman *et al.*, 1997). In *WT* anthers, the callose wall is clearly present around and between microspores at the tetrad stage (Fig 3.9), whereas in the *mpk4* mutant, no clear intersporal callose staining can be detected at any pollen developmental stage (Fig 3.9). In some instances, faint furrows can be observed which may mark attempted division of the MMC cytoplasm. Taken together, these data indicate that *mpk4* anther primordia can develop normally to the stage of producing MMCs, but fail to form an intersporal callose wall during meiotic cytokinesis.
Fig 3.8 Toluidine Blue staining of transverse sections of anther locules.

Anther developmental stages are indicated at the left side of the pictures, according to Sanders et al. (1999). E, epidermis; En, endothecium; ML, middle layer; T, tapetum;
MMC, microspore mother cell; MC, meiotic cell; Tds, tetrads; MSp, microspores. All the images were acquired at the same magnification.

Fig 3.9 Aniline Blue staining of callose wall in Ler and \textit{mpk4} tetrads.

Scale bar = 11 \text{\textmu}m.
3.3.2.3 *mpk4* male gametes can proceed through mitosis and mitotic cytokinesis

In *WT* anther locules, when microspores are freed from the tetrads, the nucleus of each microspore moves to one side of the cell and undergoes asymmetric pollen mitosis I (PMI) to form a vegetative nucleus and a generative nucleus. The newly formed generative cell is separated from the vegetative cytoplasm by a transient callose wall (Fig 3.10 A & B), which is fused at its margins with the vegetative cell wall. In the *mpk4* mutant, although the haploid microspores have not separated completely, they can still execute PMI normally. As a result of the prior failure of meiotic cytokinesis, however, PMI yields various shapes of microgamete that contain multiple generative cells (Fig 3.10 C-F), all of which are properly walled-off from the vegetative cytoplasm.
**Fig 3.10 Transmission electron microscopy of developing mpk4 pollen.**

TEM of Ler WT (A & B) and mpk4 (C-F) microspores at the bicellular stage after PMI. (B) is a close-up image of (A). Note that mpk4 microspores contain more than one generative cell, but that these are properly walled-off from the vegetative cell cytoplasm. VC: vegetative cells; n: nuclei of the vegetative cells; GC: generative cells. Black arrow heads indicate the callose wall. Scale bar = 10 µm in all except (B), and 2 µm in (B).
After PMI, the generative cell undergoes a second round of mitosis (PMII) to produce two identical germ cells, resulting in a classic tricellular structure in Arabidopsis WT pollen. The three nuclei can be visualized by DAPI staining, which shows the vegetative nucleus as a more diffuse organelle, whereas the two germ cell nuclei appear bright and compact (Fig 3.11 A). When I quantified the nuclear number of WT pollen by microscopy, 88% of the pollen grains were found to possess the typical tricellular structure, while ~10% appeared to contained one germ cell and one vegetative cell (Fig 3.11 B), although this reduced class may consist of pollen grains in which the two germ cell nuclei were aligned such that one of them could not be detected. When grown under the same conditions, less than 40% of the pollen grains in the mpk4 mutant contained the expected tricellular structure (Fig 3.11 B). Instead, mpk4 pollen grains often contained larger numbers of nuclei (Fig 3.11 A), whose frequency distribution displayed peaks attributed to (2S+1V) and (4S+2V) nuclei, in addition to a few (6S+3V) nuclei (Fig 3.11 B). The observation that mpk4 pollen grains often contain additional sets of nuclei at varying ploidy levels suggests either that some of the vegetative nuclei in the mpk4 mutant can proceed through post-meiotic development while others cannot, or that the attempts to complete meiotic cytokinesis in the mpk4 background might sometimes be successful at later developmental stages (Spielman et al., 1997). Consistent with the latter possibility, we observe portions of exine-like and intine-like cell walls within near-mature mpk4 pollen (Fig 3.12), even though mature angiosperm pollen do not possess internal cell walls. Whether these internal exine walls are fully enclosed in the cytosol or linked with the external exine is unclear, but the internal intine-like walls do appear to be connected with the peripheral intine wall (Fig 3.12).
Fig 3.11 Numbers of nuclei in mature pollen grains.

A. DAPI staining of Ler WT and *mpk4* mature tricellular pollen grains. B. Quantitative analysis of nuclear abundance in Ler WT and *mpk4* pollen.
Fig 3.12 Transmission electron micrographs of mature *mpk4* pollen.

Mature *mpk4* pollen containing internal pollen walls. White arrowheads indicate internal exine wall and black arrowheads indicate internal intine wall. Right panel is the close up images of the left panel. Bar=10 µm in left panel and 2 µm in the right panel.
3.3.2.4 Upstream activators of MKK6-MPK4 cascade in meiotic cytokinesis

It has been reported that \textit{anq1/mkk6-1} mutant Arabidopsis plants form enlarged round pollen grains (Soyano \textit{et al.}, 2003), implying that MKK6 could be the upstream MAP kinase kinase of MPK4 in pollen formation. I identified another T-DNA knockout allele of MKK6 (SALK\_117230, designated \textit{mkk6-2}) and confirmed that the phenotype is caused by the loss of function of MKK6 (Fig 2.4). The \textit{mkk6-2} mutant plants also produce malformed pollen with obvious cell division defects (Fig 3.13 A). Consistent with this, GUS activity was detected primarily in pollen grains in floral organs from both \textit{Promoter\textsubscript{MKK6}::GUS} lines and \textit{Promoter\textsubscript{MPK4}::GUS} lines (Fig 3.13 B). Previous research had identified male sterile alleles \textit{tes/stud/atnack2} that produce enlarged pollen grains with irregular germination apertures (Fig 3.13 A), which phenocopies the pollen grains of the \textit{mpk4} mutant (See Discussion) (Hulskamp \textit{et al.}, 1997, Oh \textit{et al.}, 2008, Spielman \textit{et al.}, 1997, Tanaka \textit{et al.}, 2004, Yang \textit{et al.}, 2003a). In this mutant, a kinesin-like protein that is required for male-specific meiotic cytokinesis in Arabidopsis, is disrupted. Interestingly, the tobacco orthologue of TES/STUD/ATNACK2 was reported earlier to be the upstream activator of a MAP kinase cascade (Nishihama and Machida, 2000, Takahashi \textit{et al.}, 2004). In light of these parallels, I postulated that TES/STUD/ATNACK2 could be the upstream kinesin that specifically regulates meiotic cytokinesis upstream of the MKK6-MPK4 cascade. To test this hypothesis, and to identify the MAPKKK(s) that might possibly connect TES/STUD/ATNACK2 and the MKK6-MPK4 module, I tested the interaction of two MAPKKKs, MEKK1 and ANP3, with the C-terminal region of TES/STUD/ATNACK2 (TES-C) (Ishikawa \textit{et al.}, 2002), and with both MKK6 and MPK4. MEKK1 was earlier reported to interact directly with MPK4 (Ichimura \textit{et al.}, 1998), and to be essential for MPK4 activation in different biological contexts (Ichimura \textit{et al.}, 2006, Nakagami \textit{et al.}, 2006, Suarez-Rodriguez \textit{et al.}, 2007). ANP3, on the other hand, is the closest structural orthologue of NtNPK1 (Jouannic \textit{et al.}, 1999). Protein-protein interaction assays showed that MPK4 can indeed interact with MEKK1 directly, as previously described, but not with ANP3, while TES-C and MKK6 both could interact with ANP3, but not with MEKK1 (Fig
3.14), These results are consistent with a model in which ANP3 serves as the link between TES/STUD/ATNACK2 and an MKK6-MPK4 module. ANP family members have earlier been shown to be required for mitotic cytokinesis (Krysan et al., 2002), and I conclude from my results that one or more ANP isoforms are likely acting as the upstream MAPKKK(s) for MKK6 and MPK4 in the context of male gametogenesis.
Fig 3.13 Phenotypic investigation of the upstream components of MPK4 in pollen formation.

A. Scanning electron microscopic views of WT Col and WS, and mkk6-2 (Col), mpk4 (Ler), tes-1 (Col), tes-4 (WS) anther and pollen grains. Arrow points to the furrow between undivided microspores. All the images were acquired at the same magnification. Scale bar = 25 µm.

B. Histochemical analysis of AtKK6 Promoter::GUS and MPK4 Promoter::GUS gene expression in flowers of transgenic Arabidopsis plants.
Fig 3.14 Protein-protein interaction between candidate upstream components of the MPK4 signalling cascade in pollen formation.

A. Yeast two-hybrid assay. Proteins of interest were fused with either Gal4 DNA binding domain (DB) or the Gal4 transactivation domain (AD) as indicated. Successful co-transformants were selected from SC-LT plates. Positive interactions were selected based on growth on SC-LT-His+3AT plates and on the more stringent SC-LT-Uracil plates. B. Protein-protein interactions detected in the Arabidopsis protoplast BiFC assay.
3.3.2.5 Possible targets of MPK4 during pollen formation

Since the *mpk4* mutant fails to form a callose wall during meiotic cytokinesis, I considered the possibility that *mpk4* could be required for callose synthesis and/or deposition. Callose is thought to be synthesized by callose synthases (CalSs) / glucan synthase-like (GSLs), which are encoded by a 12-member family in the Arabidopsis genome (Hong *et al.*, 2001). GSL1 and GSL5 have been shown to be specifically involved in meiotic cytokinesis, since the tetrads from the *gsl1-1/+ gsl5-2/gsl5-3* genotype lack an intersporal callose wall, similar to the *mpk4* phenotype (Enns *et al.*, 2005). Other members of the family (GSL8 and GSL10) function in microspore mitosis (Huang *et al.*, 2009, Toller *et al.*, 2008) or in exine formation (GSL2/Casl5) (Dong *et al.*, 2005). However, RT-PCR analysis of eight GSLs, revealed no reduction in their expression level in *mpk4* mutant buds (Fig 3.15 A). While MPK4 therefore does not appear to control expression of callose synthases in floral tissue, it is still possible that some other steps in callose biosynthesis or deposition require MAP kinase phosphorylation of effector proteins.

MAP kinase signalling has been previously shown to directly participate in plant mitotic cytokinesis, insofar as one member of the MAP65 family of microtubule-associated proteins, MAP65-1, can be phosphorylated by MPK4 (Beck *et al.*, 2010). However, there are eight additional members of the MAP65 gene family annotated in the Arabidopsis genome (Hussey *et al.*, 2002), and expression of one of them, MAP65-9, is primarily restricted to the pollen and stamen (Smertenko *et al.*, 2008). I postulated that MAP65-9, which also contains a putative MAP kinase phosphorylation site (PXS/TP) in its C-terminus, might represent a pollen-specific MAP65 target for the ANP3-MKK6-MPK4 cascade. Consistent with this, incubation of recombinant GST-MAP65-9 protein with activated MPK4 demonstrated that MAP65-9 could serve as an MPK4 substrate (Fig 3.15 B). On the other hand, characterization of a homozygous *map65-9* T-DNA insertion mutant (GABI_605A03) did not reveal a pollen-defective phenotype similar to that of *mpk4* (data not shown). If MAP65-9 is one of the targets of the male meiotic cytokinesis cascade, therefore, its absence might be
complemented by the action of other, more generally expressed, members of the MAP65 family.

**Fig 3.15 Possible targets of MPK4.**

**A.** Expression of callose synthase (*CalS*) / glucan synthase-like (*GSL*) genes in *WT* and *mpk4* mutant inflorescence. RNA was isolated from the top of the inflorescence containing both flowers and buds and 1 µg was used for reverse transcription. Gene-specific primers were used to detect the GSL mRNAs in the samples. Note that no GSL9 mRNA could be detected after 30 cycles of PCR in either WT or *mpk4* inflorescence samples.

**B.** Phosphorylation of MAP65-9 by MPK4 in *in vitro* phosphorylation assay. Empty arrowheads indicate the position of GST-CAKK6 on the gel; Stars indicate the position of MPKs; Black arrowhead indicates the position of GST-MAP65-9.
3.4 Discussion

3.4.1 Paralogous genes and MAP kinases

Genome duplication has shaped the structures of eukaryotic genomes over time, creating polyploidy. Genomic sequence analysis has provided evidence for up to three polyploidization events during the evolutionary history of the model plant, *Arabidopsis thaliana* (Henry *et al.*, 2006). The most recent genome duplication is estimated to have occurred around 30-35 Myr ago (Bowers *et al.*, 2003, Ermolaeva *et al.*, 2003). In addition to whole genome (WG) duplication, gene duplication can happen at a relatively smaller scale, through segmental duplication of chromosome regions, tandem gene duplication, and duplicative retroposition (Zhang *et al.*, 2010b), all of which can generate paralogous gene pairs (Cui *et al.*, 2006). While most duplicated genes lose their original functions, some genes gain new functions and/or expression patterns (neofunctionalization), or subdivide their functions in a dose-dependent manner (subfunctionalization).

The plant MAP kinase gene families experienced gene duplication events at different levels and these have expanded the family size greatly. Paralogous gene pairs are apparent from the phylogenetic tree (Hamel *et al.*, 2006, Ichimura *et al.*, 2002). Studies of these paralogous gene pairs provide further understanding of the functional overlapping and subdivision within this family. For instance, MPK3 and MPK6 represent the best characterized paralogous MAP kinases, and they are functionally redundant in a wide spectrum of aspects, such as ethylene biosynthesis (Han *et al.*, 2010, Liu and Zhang, 2004), abiotic stress response (Liu *et al.*, 2010), disease resistance (Beckers *et al.*, 2009, Ren *et al.*, 2008, Zhang *et al.*, 2007a), and plant development, including stomata patterning, floral organ abscission, anther and ovule development (Cho *et al.*, 2008, Hord *et al.*, 2008, Wang *et al.*, 2008a, Wang *et al.*, 2007). While *mpk3* and *mpk6* single mutants are largely comparable to WT, the double mutant is embryo lethal. Nevertheless, MPK3 and 6 have retained/evolved specialized functions that are not shared with the other parologue. For example, it is reported that MPK3...
phosphorylates the transcription factor VIP1 in response to *Agrobacterium* transfection (Djamei *et al.*, 2007), and that MPK6 localizes to distinct spots in the preprophase band, phragmoplast, plasma membrane and trans-Golgi network, and facilitates cell division in Arabidopsis roots (Müller *et al.*, 2010). A large scale screening of MPK3 and MPK6 phosphorylation substrates using a protein microarray containing 1690 Arabidopsis proteins identified 48 potential substrates for MPK3 and 39 for MPK6, and the two kinases shared 26 substrates in common (Feilner *et al.*, 2005). At the MKK level, MKK2 shares functions in disease resistance response and ROS signalling with its paralogous gene, MKK1 (Gao *et al.*, 2008, Pitzschke *et al.*, 2009, Qiu *et al.*, 2008a), but MKK2 also facilitates the cold and drought stresses by itself (Teige *et al.*, 2004). MKK4 and MKK5 represent another set of paralogous MKK genes sharing overlapping functions in stress response and plant development (Cho *et al.*, 2008, Wang *et al.*, 2007). Despite these interesting observations, limited effort has been made to understand the full extent to which paralogous gene pairs function redundantly or independently.

*MPK4* and *MPK11* are another set of paralogous genes in the Arabidopsis MAP kinase family whose relationship is poorly understood. While MPK4 was intensively studied, especially by plant pathologists in the disease resistance field, MPK11 is one of the ‘untouched MPKs’. My initial computational analysis showed no evidence for this pair being duplicated in any of the whole genome duplication events, suggesting that they were probably duplicated in a more recent small scale duplication event.

### 3.4.2 Compensatory strategy

During evolution, duplicated genes provide a rich reservoir for evolving new gene functions. While one copy of most duplicated genes is ultimately lost, some become neofunctionalized, and others are subfunctionalized (Hahn, 2009). *MPK11* and *MPK4* are duplicated gene pair, and *MPK11* is neofunctionalized by adopting a new expression atlas. *MPK11* transcript is actively regulated by various plant hormones and stress signals, suggesting that MPK11 might be
specialized at particular developmental stages and in stress signalling. The fact that MPK11 expressed under the control of MPK4 promoter cannot compensate for the loss of MPK4 indicates that the two paralogous genes are not functionally interchangeable. Interestingly, in the mpk4 mutant background, elevated MPK11 transcription was evident, as assessed by RT-PCR, but it is likely that the increased SA accumulation in the mpk4 mutant background induced the increased MPK11 expression. However, it is also possible that the mpk4 mutant might increase the level of MPK11 in an attempt to compensate for the loss of MPK4, which would imply that MPK11 might be partially sustaining the function of MPK4. Such a compensatory effect was also observed in another set of paralogous genes, NtSIPK and NtWIPK, in tobacco. Over-expression of NtSIPK rendered the plant sensitive to ozone, similar to the silencing of NtSIPK, in which higher and prolonged NtWIPK activation was also detected (Samuel and Ellis, 2002). In Arabidopsis, silencing of the NtSIPK orthologue, MPK6, also renders the plants hypersensitive to ozone and leads to a similar extended activity of the NtWIPK orthologue, MPK3. Reciprocally, in the mpk3 loss of function mutant, MPK6 activity is prolonged after ozone treatment (Miles et al., 2005). These observations suggest that a compensation strategy in response to negative mutations, operating on either the transcriptional or (post-) translational level, for paralogous gene pairs, might be a common theme in plant biology. Finally, my inability to identify mpk4/mpk11 double mutant progeny suggests that MPK11 may partially share overlapping function, and/or that it’s function is essential for the survival of mpk4. However, considering the abnormal segregation ratio observed from the progeny of a single double heterologous plant, it is also likely that the mpk11 mutation locus and the mpk4 mutation locus were somehow located on the same chromosome. In fact, a recent publication indicated that the T-DNA insertion in mpk11 has been translocated to Chromosome 4, where MPK4 resides (Clark and Krysan, 2010).

3.4.3 MPK4 and MPK11 share high level of sequence similarity but are not functionally interchangeable

Ancient polyploidy event in plants have frequently created protein families with
multiple members sharing high level of sequence similarity (Moore and Purugganan, 2005). Proteins from the same family typically share similar functions thus ensuring that the negative mutation of one essential gene may not cause lethality. However, in some cases, these closely related members of a gene family may be differentially expressed, and display unequal redundancy. To examine if the protein functions are interchangeable, ‘promoter swap’ experiments have been used to try to restore the mutant phenotype. For example, in the regulation of the shoot apical meristem during development, CLV3 was proposed to bind to its receptor CLV1, which then forms a complex with another leucine-rich repeat receptor-like protein (RLP) CLV2. Together, this complex is required for maintaining the stem cell population (Barton, 2010). In Arabidopsis, CLV3 and CLV2 each belong to multi-member protein families, both of which contain members sharing highly conserved domains and/or sequence similarity. It was reported that CLE40, expressed under the control of the CLV3 promoter, can rescue the clv3 mutant in almost all its defects (Hobe et al., 2003). Moreover, representatives from different groups of receptor-like proteins that relates to CLV2 were selected to be fused with the CLV2 promoter and transformed into the clv2 mutant. While AtRLP2 and AtRLP12 expressed in this fashion were able to rescue the mutant phenotype, AtRLP30 and AtRLP38 did not, indicating that AtRLP2 and AtRLP12 are functionally equivalent to CLV2 (Wang et al., 2010a). Surprisingly, none of these four receptor-like proteins shares more than 30% amino acid sequence identity with CLV2.

In the auxin transduction pathway, the receptor, TIR1, is conserved in all land plants and studies showed that the members of this family, TIR1, AFB1, AFB2, and AFB3 are all required for auxin response. While TIR1, AFB2, and AFB3 contribute equally to the response, AFB1 has a less important role (Dharmasiri et al., 2005). To understand their functional relationship, AFB1 and AFB2 were expressed in tir1 plants under the control of the TIR promoter. It was found that these tranregenes were unable to rescue the altered sensitivity to auxin in the tir mutant, even though they share more than 60% of sequence identity (Parry et al., 2009). These studies suggest that 1) proteins of the same family may have over-
lapping function in the same pathway, but they may not be functionally equivalent; and 2) whether two proteins are functionally interchangeable does not correlate with the percentage of sequence similarity. My study represents another example, where two paralogous kinases, sharing amino acid sequence identity as high as 88.3%, are not functionally interchangeable. Although MPK11 is able to interact with, and be phosphorylated by, MKK6 and can then phosphorylate MBP, its enzyme activity is much lower than that of MPK4 when tested in the in vitro kinase assay. If MPK11 does not have enzymatic activity comparable to that of MPK4, it may not be able to functionally compensate for the loss of MPK4 even when its expression is under the control of the MPK4 promoter.

3.4.4 MPK4 functions in male meiotic cytokinesis

The process of pollen formation requires the coordinate action of numerous genes responsible for the establishment of stamen identity, for cell division and differentiation during anther and pollen development, and for executing programmed cell death in specific tissues to allow release of the mature pollen grains (Ma, 2005). MPK4 is already known to play a role in disease resistance (Andreasson et al., 2005, Brodersen et al., 2006, Gao et al., 2008, Petersen et al., 2000, Qiu et al., 2008a, Suarez-Rodriguez et al., 2007), and in this context, one feature of the mpk4 mutant phenotype is a massive increase in constitutive salicylic acid (SA) accumulation, accompanied by suppression of jasmonate-induced gene expression (Petersen et al., 2000).

Arabidopsis mutants defective in jasmonate biosynthesis or signalling are known to display male sterility (Feys et al., 1994, Zhao and Ma, 2000). However, they exert this effect by delaying anther dehiscence and inhibiting anther filament elongation (Zhao and Ma, 2000), which is distinct from the phenotype I observed in the mpk4 mutant. In addition, the signal transduction mutant, snc1, which also displays a dwarf phenotype and constitutively accumulates high levels of SA (Li et al., 2001), shows normal levels of fertility (X. Li, personal communication) and produces pollen grains that are a little smaller than WT (Fig 3.16). These data suggest that the mpk4 male sterility is caused by neither JA insensitivity nor high
levels of SA accumulation.

Fig 3.16 Comparison of the pollen grain phenotypes of Col and snc1.

Images were acquired at the same magnification.

In the early stages of anther development, the anther primodium forms four archesporial cells, each of which then develops into a primary sporogenous cell and a primary parietal cell (Scott et al., 2004). Mutations that affect cell differentiation or cell fate determination at this stage lead to male sterility. For example, two paralogous Arabidopsis MAP kinases, MPK3 and MPK6, are required for normal male gamete formation, since the mpk6\(^{-/-}\) mpk3\(^{+/-}\) mutant often fails to develop all four locules in a given anther, and as a result, produces fewer viable pollen than WT (Hord et al., 2008). At the same time, this mutant is also female sterile as a result of arrest of late stage cell division in the ovule integuments (Wang et al., 2008a). Other genes, such as SPOROCYTELESS/NOZZLE (SPL/NZZ), EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS (EMS1/EXS), and TAPETUM DETERMINANT1 (TPD1), are essential for the formation of sporogenous cells or the somatic tapetum cells (Canales et al., 2002, Schiefthaler et al., 1999, Yang et al., 2003b, Yang et al., 1999, Zhao et al., 2002), and mutations at these loci result in empty anther locules. The mpk4 mutant anther, however, is able to produce a functional tapetum and apparently normal MMCs (Fig 3.8), suggesting that MPK4 does not play an essential role in the early stages of microsporogenesis.

After meiosis, individual haploid microspores go through two rounds of mitosis.
Mutations in *GEMINI POLLEN 1/MICROTUBULE ORGANIZATION 1* (GEM1/MOR1) affect the unequal division of mitosis I (Park *et al.*, 1998, Twell *et al.*, 2002), while *duo* mutations block mitosis II by terminating the generative cell cycle, resulting in pollen grains with one vegetative cell and one generative cell (Brownfield *et al.*, 2009, Durberry *et al.*, 2005). The plasma membrane-localized MAPKKKs, MAP3Kϵ1 and MAP3Kϵ2A, also appear to act after pollen mitosis I, and mutations in the corresponding genes result in non-viable pollen (Chaiwongsar *et al.*, 2006). My data, on the other hand, demonstrate that MPK4 is not required for either mitotic cytokinesis or for cell lineage determination, but rather, plays a specific role in meiotic cytokinesis during pollen development.

### 3.4.5 MPK4 in somatic cytokinesis

The severe dwarfism phenotype of the *mpk4* mutant (Petersen *et al.*, 2000) suggests that MPK4 might also play a role in somatic cytokinesis, and a recent study found MPK4 to be physically associated with cortical microtubules and to regulate their bundling (Beck *et al.*, 2010). Very recently, MPK4 was indeed shown to be required for somatic cytokinesis (Kosetsu *et al.*, 2010). These reports are consistent with the ubiquitous expression of *MPK4* transcripts (Fig 2.8 A & B). My study, however, emphasizes the subtle distinction between the degrees to which MPK4 activity is essential for different cellular processes. For example, in order to form the MMCs, the anther primordia have to successfully complete a series of defined cell divisions and differentiation, and the *mpk4* mutant anther is able to do so. MPK4 thus appears to be essential for male meiotic cytokinesis, and also for somatic cytokinesis in some, but not all, tissues. Such specificity could potentially result from complementation in certain tissues by other distinctively-localized AtMPKs that are functionally redundant with MPK4. Alternatively, MPK4 might recruit upstream activators and/or downstream targets that are uniquely expressed in those tissues/cells.

### 3.4.6 *mpk4* phenocopies *tes/stud/atnack2*

Consistent with a ‘recruitment specificity’ model is the observation that mutation of an Arabidopsis kinesin, *TES/STUD/AtNACK2*, distinctively results in a pollen...
phenotype marked by a defect in male meiotic cytokinesis, (Hulskamp et al., 1997, Oh et al., 2008, Spielman et al., 1997, Tanaka et al., 2004, Yang et al., 2003a). Like mpk4, tes/stud/atnack2 floral organs form normal sporogenous cells and peripheral supporting tissues, indicating that neither gene is required for early anther development or for the generation of MMCs. In both mutants, MMCs fail to produce an intersporal callose wall, but the mature pollen grains contain irregular internal cell walls. Although meiotic cytokinesis is not completed in either mutant genotype, mitotic cytokinesis appears to proceed normally within the multi-nucleate microgametophyte, resulting in over-size mature pollen grains that contain more than one set of tricellular structures. Mutants contain increased number of nuclei within pollen grains can germinate more than one pollen tubes (Chen et al., 2009). But mpk4 and tes/stud/atnack2 pollen grains germinate to produce a single pollen tube (Fig 3.17), despite containing extra sets of nuclei. The multi-nucleate microspores thus still retain the ability to complete asymmetric mitotic cell division (Spielman et al., 1997). Homozygous mutants of either locus are able to produce only a few viable seeds, together with many dead/empty ones. It is clear, therefore, that mpk4 phenocopies tes/stud/atnack2, and that TES/STUD/AtNACK2 and MPK4 are very likely to operate in the same pathway in regulating meiotic cytokinesis.

Fig 3.17 Pollen germination and tube growth of WT and mpk4.
Images were acquired at the same magnification.

3.4.7 TES/STUD/AtNACK2 is upstream of the MAP kinase cascade ANPs-MKK6-MPK4

It has been proposed that the tobacco kinesin, NtNACK, operates upstream of
the so-called MAP NP-Q-RK cascade to modulate phragmoplast expansion during cytokinesis (Sasabe et al., 2006, Takahashi et al., 2004). NtNACK has two orthologues in Arabidopsis; AtNACK1/HINKEL, which is involved in somatic cytokinesis and is essential for embryogenesis (Strompen et al., 2002), and AtNACK2/TES/STUD. Since mpk4 essentially phenocopies tes/stud/atnack2 in pollen development, I propose that TES/STUD/AtNACK2 acts upstream of a MAP kinase cascade that involves MPK4. The demonstration that the C-terminus of TES/STUD/AtNACK2 (Tes-C) (Ishikawa et al., 2002) can interact with one of the cytokinesis-related AtMAPKKK/ANPs, ANP3 (Krysan et al., 2002), that ANP3 interacts with M KK6 in the protoplast BiFC assay (Fig 3.14 B), and that MKK6 can interact with and phosphorylate MPK4, supports the inclusion of ANP3 and MKK6 in a meiotic cytokinesis-associated signalling module consisting of TES-ANP3-MKK6-MPK4. Notably, the anq1/mkk6-1 mutant also produces malformed and enlarged tetrad-like pollen grains (Soyano et al., 2003), although the anp3 mutant does not (Fig 3.18). Other ANP family members may therefore compensate for loss of ANP3 function in male meiotic cytokinesis signalling.

Fig 3.18 Comparison of mature pollen sizes of different genotypes.

For each genotype, n=10, 16, 10, 10, 9, 44, 27, and 60, respectively. Stars indicate significant different compare with the corresponding WT. Specifically, mpk4 is compared to Ler, whereas the anp mutants are compared to WS. All the images were acquired at the same magnification.
Based on the original tobacco NtNP-Q-RK model, the Arabidopsis orthologue of NtNRK is predicted to be MPK13, rather than MPK4. However, the T-DNA insertion allele of MPK13 does not exhibit any morphological phenotype (SALK_130193), perhaps because the knockout allele disrupts the long splice variant of MPK13, but not the short variant. While MPK13 RNAi lines in which MPK13 expression is partially suppressed display reduced lateral root formation (Fig 2.11), which would be consistent with a role in somatic cytokinesis, they are not male sterile. Since loss of function of MPK4 alone causes the meiotic cytokinesis defect, it appears that MPK13 is unlikely to share overlapping functions with MPK4, at least in pollen meiotic cytokinesis.

Two other Arabidopsis MKKs, MKK1 and MKK2, are also known to interact with MPK4 (Lee et al., 2008), and the mkk1/mkk2 mutant is severely dwarfed (Gao et al., 2008, Qiu et al., 2008b). However, the double mkk1/mkk2 mutant displays premature senescence and is ultimately lethal. In addition, the MAPKKK acting upstream of MKK1/2, MEKK1, does not interact with TES/STUD/AtNACK2. It is therefore more likely that the MEKK1-MKK1/2-MPK4 pathway regulates disease and stress responses (Gao et al., 2008, Pitzschke et al., 2009, Qiu et al., 2008b, Suarez-Rodriguez et al., 2007), while the TES/STUD/AtNACK2-ANPs-MKK6-MPK4 module regulates cytokinesis.

### 3.4.8 MPK4 and its targets in meiotic cytokinesis

The MAP kinases have a long history of being associated with microtubule organization, since the first MAPK was identified as a Microtubule-Associated Protein (MAP) kinase (Ray and Sturgill, 1987). Both the abundance and phosphorylation status of the Arabidopsis microtubule-associated protein, MAP65-1, have been shown to be affected in mpk4 and anp2/anp3 mutants (Beck et al., 2010), while its tobacco homologue, NtMAP65-1, can be phosphorylated by both NtNRK1 and NtCDKs (Sasabe et al., 2006). Since the phosphorylation status of NtMAP65-1 influences both MT organization and cell cycle progression, interference with the ability of the relevant kinases to modify
MAP65-1 homologues would be predicted to affect these same processes. On the other hand, there is no direct genetic evidence that loss of MAP65-1 results in defects in MT organization and/or cytokinesis. Similarly, while I have shown that the pollen-specific MAP65 isoform, MAP65-9, can be phosphorylated by MPK4, as the putative end-point of a meiotic cytokinesis signalling cascade, loss of function at this locus in map65-9 plants produced no apparent defects in fertility or pollen grain phenotype.

It is noteworthy that whereas mpk4 pollen specifically fails to synthesize an intersporal callose wall, mpk4 rosette leaves were earlier reported to display increased levels of callose accumulation and β-1,3-glucan synthase activity (Ostergaard et al., 2002). My RT-PCR analysis of floral tissue also confirmed that expression of some GSLs is elevated in the mpk4 mutant (Fig 3.15). MPK4 thus appears to influence callose formation in opposing ways. In the context of disease resistance, it serves as the terminal MPK in the MEKK1-MKK1/2-MPK4 cascade, but in the context of male meiotic cytokinesis, MPK4 operates within a specific TES/STUD/AtNACK2-ANPs-MKK6-MPK4 cascade. In the latter role, MPK4 may facilitate callose deposition by regulating MT dynamics and/or vesicle trafficking associated with the formation of the mini-phragmoplast.
CHAPTER 4 GENERAL DISCUSSION
4.1 Summary and Synthesis

4.1.1 MKK6 is an essential MKK for plant development in Arabidopsis

MAP kinases are versatile signalling components that regulate a wide range of cellular process in response to both exogenous and endogenous signals. They are activated through the activity of MPK kinases, and null mutant analysis has shed light on the functions of several of the Arabidopsis MPK kinases. MKK1 and MKK2 form a paralogous pair, and the loss of function mutant of either MKK1 or MKK2 does not result in an obvious morphological phenotype under normal growth conditions. When treated with ABA or bacterial elicitor flagellin, however, mkk1 has altered sensitivity compared to WT (Meszaros et al., 2006, Xing et al., 2009), whereas mkk2 is hypersensitive to cold and salt stress (Teige et al., 2004). The double mkk1/mkk2 mutant, on the other hand, is severely dwarfed and ultimately seedling lethal, suggesting that MKK1 and MKK2 are functionally redundant in plant development (Gao et al., 2008, Qiu et al., 2008b). The mkk3 mutant, in which the T-DNA is inserted between the kinase domain and the NTF domain, displayed hypersensitivity to JA and to bacterial infection, but otherwise the mutant behaves comparable to WT without treatment (Doczi et al., 2007, Takahashi et al., 2007). No null alleles of MKK4 and MKK5 have been identified, but MKK4 RNAi and MKK5 RNAi lines were generated, and the double RNAi lines produce unusual clustered stomata (Wang et al., 2007). Originally identified as an MKK7 over-expression line, the bud1 mutant produces extremely branched shoots due to defects in polar auxin transport (Dai et al., 2006). On the other side, although introgression of an antisense MKK7 construct restores the bushy phenotype in bud1, the suppression of MKK7 expression in WT background did not cause any visible phenotype under normal growth conditions (Dai et al., 2006). Similarly, mkk9 mutant plants only have a subtle delay in leaf senescence (Zhou et al., 2009). Detached leaves senesce greatly later than WT, which is consistent with the fact that MKK9 overexpression lines showed increased ethylene production and early leaf senescence (Xu et al., 2008, Zhou et al., 2009).
Null mutants of MKK6 are severely dwarfed and stressed (Fig 2.4) (Takahashi et al., 2010). At the cellular level, mkk6 plants are not able to complete cytokinesis in some tissues, but display ectopic cell division in other tissues. Cell elongation is suppressed to a certain degree (cortex cells, Fig 2.6 A&B), while unexpected cellular protrusion is also evident (root hair, Fig 2.6 A). None of the reported AtMKK single mutants, either null allele or repression transgenic line, have developmental defects as severe as those observed in mkk6 plants, indicating that MKK6 plays a distinct and essential role in plant development. However, when paralogous AtMKKs involved in central processes share overlapping functions, the plant cannot survive when both are absent. Since the mkk6 mutant is not completely lethal, the presumption is that there must be other MKK(s) that is/are able to coordinate cell division to a certain extent when MKK6 is knocked out, or alternative machinery exists that allows the cell to execute cytokinesis while bypassing the MKK6 MAP kinase cascade.

4.1.2 MKK6 has multiple target MPKs and is required for MT organization

The plant cytoskeleton consists of two major types of protein polymers, actin filaments (AFs), which are double helical strands made of a globular protein called actin, and microtubules (MTs), which are hollow tubes made of α and β tubulins (Petrasek and Schwarzerova, 2009). These structures play important roles in supporting the cell shape, determining the direction of cell expansion, transporting intracellular components, and managing cell division. Microtubules, in particular, are highly organized and dynamic, characteristics which are modulated by a variety of interacting proteins, such as MAPs (Hamada, 2007), motor protein kinesins (Lee and Liu, 2004), and MAP kinases (Sasabe et al., 2006, Walia et al., 2009).

It has been proposed that ANQ1/MKK6 is required for cytokinesis by controlling the phosphorylation of Microtubule-Associated Protein 65 and thereby regulating phragmoplast dynamics (Sasabe et al., 2006, Soyano et al., 2003). My study of the phenotype of MKK6 RNA interference lines and a knockout allele (Fig 2.5 & 11) support a role for MKK6 in cell division. Specifically, my data show that the
MKK6-MPK13 pathway is required for lateral root formation, while a MKK6-MPK4 module is required for meiotic cytokinesis. Very recent studies have demonstrated that MPK4 regulates cytokinesis in leaves and roots, as well, because the strong loss of function alleles of \textit{mpk4} in the Columbia background display incomplete cell wall formation in these tissues (Beck \textit{et al.}, 2010, Kosetsu \textit{et al.}, 2010). In addition to its importance for successful cytokinesis, MKK6 also seems to influence MT dynamics and organization in general. The fat and short root cortex cells in \textit{mkk6} plants suggest that MKK6 might be required for the parallel alignment of the anticlinal cortical MT arrays. Beck \textit{et al.} (2010) proposed, based on phenotypic analysis, molecular interactions, and enzymatic activity, that MPK4 and ANPs function in organizing cortical microtubule bundling, and that these two kinases might be bridged by MKK6. Moreover, the branched root hairs observed in both \textit{mkk6} (Col) and \textit{mpk4} (Col) mutants further imply that 1) the MKK6-MPK4 cascade is not functionally restricted to meiotic cytokinesis, or somatic cytokinesis; and 2) they have a broader effect in MT organization across a range of cell types. Taking together the cellular evidence that MPK4 is localized to the newly formed cell plates (Kosetsu \textit{et al.}, 2010), and that cortical MTs are more strongly bundled in \textit{mpk4} (Col) (Beck \textit{et al.}, 2010) cells, it appears that MPK4 is a major target of MKK6 in modulating microtubules dynamics, including roles in cortical microtubule behaviour, phragmoplast formation during somatic cytokinesis, and radial microtubule and/or mini-phragmoplast function in dividing pollen mother cells.

My work, as well as the results of others also reveals a molecular linkage between MKK6 and MPK6 (Lee \textit{et al.}, 2008, Popescu \textit{et al.}, 2009). With the recent proposition that MPK6 also participates in cytokinesis probably by delivering vesicle components, and the observation that the \textit{mpk6} exhibits a root tip meristem ectopic cell division phenotype similar to that of the \textit{mkk6-2} mutant (Fig 2.7) (Müller \textit{et al.}, 2010), MKK6 might be the upstream of MPK6 in controlling the vesicle trafficking and/or ethylene homeostasis (Fig 4.1). In all cases, the targets of the MAP kinase cascades seem to be the cell plate formation machinery involving microtubule functions, either phragmoplast.
expansion, or cell wall material transportation.

4.1.3 Signalling specificity

Given that MKK6 widely affects different parts of the plant, and in different cell types, and that the MPKs outnumber MKKs in Arabidopsis, it is not surprising that four AtMPKs are found to be able to interact with and be phosphorylated by MKK6. It seems to be a common phenomenon for AtMKKs and AtMPKs that one AtMKK can have multiple AtMPK targets in distinct pathways. For example, MKK3 phosphorylates MPK6 in response to JA (Takahashi et al., 2007), and it interacts with MPK7 in the disease resistance pathway (Doczi et al., 2007). Intriguingly, though, that even though the Arabidopsis MPK family has expanded its size greatly, there is still a high level of multifunctionality involving particular AtMPKs, such as MPK4, MPK3, and, most dramatically, MPK6. The latter kinase seems to be a target of almost every MKK that has been closely studied to date. MKK1-MPK6 regulates ABA sensitivity and biosynthesis (Xing et al., 2008), while the MKK2-MPK6 module acts in responses to cold and salt stress (Teige et al., 2004). The MKK3-MPK6 cascade negatively regulates JA signalling (Takahashi et al., 2007), and MKK4/MKK5-MPK6 is involved in cell differentiation during stomata development, and cell separation during floral abscission (Cho et al., 2008, Wang et al., 2007). MKK9-MPK6 is required for ET biosynthesis and timely leaf senescence (Xu et al., 2008, Zhou et al., 2009).

One of the fundamental questions to ask about the plant MAP kinase network is how the signalling specificity is achieved. One apparent mechanism is the tissue- or cell type-specific expression of the components. This mechanism is evident for the MKK6-MPK13 cascade, which are co-regulated developmentally (Fig 2.8) and in a cell cycle dependent manner (Menges et al., 2008), supporting their roles in cell proliferation (Fig 4.1). However, for genes such as MPK4 and MPK6, which are ubiquitously expressed in almost all tissues, plant cells need alternative regulatory machinery to control specificity. One likely explanation is that these ubiquitous MAP kinases differentially recruit upstream activators and/or downstream effectors that only function in particular tissues/cells.
Although the MAP kinase cascade ANPs-MKK6-MPK4 does not function exclusively in meiotic cytokinesis, the upstream AtNACK2/TETRASPORE/STUD seems to be a kinesin that only functions in microspore mother cells, based on genetic analysis. In somatic cell division and, probably, cortical microtubule organization, the same ANP-MKK6-MPK4 cascade might operate downstream of the closely related AtNACK1/HINKEL kinesin (Fig 4.1), which has a more profound impact on cytokinesis early in embryogenesis (Strompen et al., 2002). Furthermore, subcellular compartmentalization may contribute to the specificity of the MAP kinase cascade as well. The tobacco NtNP-Q-RK cascade components localize to the cell plate in dividing cells (Nishihama et al., 2001, Soyano et al., 2003, Takahashi et al., 2004), thereby establishing a relatively high enzyme-substrate concentration. In addition, MPK6 can be observed as pinpoint distinct spots along the PPB and cell division plates, and is associated with vesicles (Beck et al., 2010), while MPK4 localizes to the expanding cell plates (Kosetsu et al., 2010). In all instances, such behavior would increase the local concentration of selected protein and therefore its enzyme activity toward its substrates.

Scaffold proteins are common regulatory components in conferring MAP kinase specificity in mammalian cells and yeast cells, and it is reasonable to imagine that plants may also make use of this mechanism. However, although MEKK1 was proposed to be functioning as a scaffold (Suarez-Rodriguez et al., 2007), there is no detailed information on how it works.

### 4.1.4 Upstream activator of M KK6

MAP kinase cascades have been shown to participate in responses to various signals in plants, but it remains unclear as how these input signals activate the kinase module. The canonical model of a MAP kinase cascade requires MKKKs to phosphorylate and activate the MKKs. Despite the expanded size of the MKKK gene family in Arabidopsis, few MKKKs have shown direct binding/activity towards MKKs. In fact, the annotations of AtMAP3Ks and AtMAP4Ks are derived from the phylogenetic and sequence relationship trees, instead of biochemical and functional properties (Champion et al., 2004). Within the two subfamilies of MAPKKK, the Raf-like MKKKs, such as CTR1 and EDR1, have not been directly
shown to activate or repress any of the defined MAP kinase cascades, despite their important roles in ethylene signalling and disease responses in plants. STE family MKKKs, however, do bind and activate MKKs, although, there are only a couple of MKKKs that have been shown experimentally to do so. One example is MEKK1 binding with MKK1 and MKK2 (Gao et al., 2008, Ichimura et al., 1998). The other is NtNPK1, which can bind with NtNQK1 (Soyano et al., 2003). I found that the Arabidopsis ANP3 can bind with MKK6, and that their interaction is mainly nuclear localized, suggesting a possible role of subcellular localization in the signal transduction. These findings are consistent with the observation that the tobacco orthologue of the ANPs, NtNPK1, contains a nuclear localization signal (NLS) at its C-terminal end and is nuclear localized (Ishikawa et al., 2002). Occasionally, a MKKK can directly bind to a MPK, and manipulate the output of the MPK, such as MEKK1 to MPK4 (Ichimura et al., 1998), and MsOMTK1 to MsMMK3 (Nakagami et al., 2004). Whether the direct binding between MKKKs and MPKs is a common scenario for plant MAP kinases remains to be examined. Intriguingly, MEKK1 can also bind directly to the promoter region of the transcription factor WRKY53, and thereby serve as a short cut in response to flagellin elicitation (Miao et al., 2007). However, I found that ANP3 does not bind to MPK4 in the yeast two-hybrid assay. Instead, I demonstrated that MKK6 can bind to both ANP3 and MPK4, and therefore serves as the missing link between ANPs and MPK4 in microtubule regulation. A previous report showed that the mekk1 mutant produces fewer lateral roots than WT (Su et al., 2007), leading to the possibility that MEKK1 might be acting upstream of the MKK6-MPK13 cascade in regulating lateral root formation. However, I did not observe a positive interaction between MEKK1 and MKK6. Conceivably, the potential in planta interaction between MEKK1 and MKK6 might require a scaffold protein, or that MEKK1 may regulate lateral root formation through other MPK pathways, while ANPs serve as the upstream activators of the MKK6-MPK13 module in the same biological context. Analysis of the lateral root phenotype of the anp2/anp3 double mutant would be very helpful to distinguish between these two possibilities.

Overall, my studies of the mkk6 pleiotropic phenotype and characterization of its
target MPKs has broadened our understanding of the role of MKK6 in modulating MT dynamics, provided new insights into the biological roles of several of the Arabidopsis MPKs, and added to our appreciation of signalling specificity, complexity, and sophisticated regulatory mechanism of plant MAP kinase cascades.

Fig 4.1 A proposed model of MAP kinase signalling network involving MKK6.
4.2 Future Directions

While my research expanded our knowledge of the MAP kinase cascade complexity, it leaves open interesting questions for future studies as well.

4.2.1 Specificity of MAP kinase cascade

As the study showed that MKK6-MPK13 is required for lateral root formation, it is unclear whether these proteins block the cell cycle progression, or only affect cytokinesis. By crossing transgenic lines with cell cycle marker genes and examining the marker genes expression, one could establish whether the cascade influences cell cycle in lateral root founder cells and lateral root primordia. In addition, immunolocalization and/or fluorescence protein labeled protein localization can determine where the kinases are concentrated at a subcellular location, which can help us understand whether and how the cascade is involved in cytokinesis.

My research suggests that two MAP kinase cascades are likely involved in cytokinesis. Although I have assigned functionality to these two cascades in lateral root formation and meiotic cytokinesis, respectively, this analysis is certainly not comprehensive. Future experiments should try to establish their functions in other specific tissue and cell types. For example, MKK6 is required for maintaining root apical meristem organization, but which MPK is required for this pathway is unknown.

The MAP kinase signalling components are involved in multiple pathways, and only partial information is available concerning the specific roles of each of the proteins. In addition, contradictory data appeared in the literatures concerning the same mpk6 mutant phenotype, which could be introduced by subtle differences in growth condition (Müller et al., 2010, Wang et al., 2010b). Comprehensive analysis of MAP kinases under the same experimental setting could therefore help disentangle the puzzle. For example, I proposed that tissue-specific expression and subcellular localization of proteins could be one of the main regulatory mechanisms. It would be informative to compare the subcellular localization of MPK6, MPK4, MPK13, and MKK6 in the same cell types,
especially dividing cells, and microspore mother cells. Similarly, root tip and lateral root physiology could be examined for the *mpk4*, *mpk6*, *mkk6*, as well as other potential MKK mutants under the same conditions. These experiments can exclude some of the kinases from a certain biological context, and thereby define components that are specific to that given pathway.

In recent years, increasing numbers of MAP kinase targets have been identified and assigned biological functions. These targets are identified either by individual one-on-one assays, or through large-scale screening. Either yeast two-hybrid library screening or protein microarray is based on direct interaction or phosphorylation. To identify indirect interactors in a protein complex, and potential scaffold proteins, *in planta* immuneprecipitation combined with mass spectrometry could be very useful. Moreover, phospho-proteomics can be advantageous in finding novel phospho-proteins that serve as *in vivo* MAP kinase targets under specific treatment conditions. It would be neat to dissect the plant and perform these screens using specific tissue types in that protein reservoirs could differ from one tissue to another.

On the other hand, even though many stimuli can activate the MAP kinase cascades, it is rarely known how a particular signal is perceived to activate the MPK cascade(s). For example, as one of the most intensively studied field, the leucine-rich-repeat (LRR) receptor-like kinase (RLK) FLS2 recognizes the bacterial elicitor flagellin and is upstream of the MEKK1-MKK1/2-MPK4 cassette (Gao *et al.*, 2008, Qiu *et al.*, 2008b, Suarez-Rodriguez *et al.*, 2007). The same signal also goes through MPK6-mediated transcription factor modification (Bethke *et al.*, 2009). But a gap in our knowledge of the linkage between the receptor and the MPK cascades remains. To identify upstream activators of a MPK module, efficient screening of mutants that have impaired MAP kinase activity upon treatment should be developed.

### 4.2.2 Negative regulators of MAP kinase cascade

Five MAP kinase phosphatases (MKPs) have been identified from the Arabidopsis genome (Kerk *et al.*, 2002). Accumulating results have suggested
that they negatively regulate the MAP kinase cascades and play important roles in phytohormone auxin and ABA signalling (Lee et al., 2009, Monroe-Augustus et al., 2003, Quettier et al., 2006, Strader et al., 2008a, Strader et al., 2008b), biotic and abiotic stress responses (Bartels et al., 2009, Lumbreras et al., 2010, Ulm et al., 2002, Ulm et al., 2001), and microtubule organization (Naoi and Hashimoto, 2004, Walia et al., 2009). Additionally, members of the PP2C-type phosphatases, AP2C1, and its close homolog, PP2C5, were shown experimentally to dephosphorylate MPK3, MPK4, and MPK6, and to regulate various hormone responses and developmental processes (Brock et al., 2010, Schweighofer et al., 2007). Nevertheless, only MPK3, MPK6, MPK4, MPK12, and MPK18 have been shown to be de-phosphorylated by one or two of the phosphatases. My study has been focused mainly on characterizing the MAP cascade components involving MKK6. Future work should aim to identify the phosphatases that negatively regulate the MKK6-related biological pathways. Since PHS1 is reported to regulate microtubule dynamics (Naoi and Hashimoto, 2004), and to dephosphorylate MPK18 (Walia et al., 2009), which is activated by MKK6 (Hua et al., 2006), it is a good candidate for testing its enzyme activity on the MKK6 related MPKs. Genetically, overexpression lines of the three known phosphatases that negatively regulate MPK4, namely, DsPTP1, PP2C5, and AP2C1, could be examined for abnormal cell division phenotypes in different tissues (Bartels et al., 2009, Brock et al., Schweighofer et al., 2007).

4.2.3 Disease resistance and development

Mutants that have altered disease resistance often have developmental problems. Constitutive disease resistant mutants sometimes display dwarfism and curving leaves. It is believed that the dwarf phenotype is caused by salicylic acid accumulation since introduction of nahG, a bacterial gene that encodes a salicylate hydroxylase which degrades SA into biologically inactive catechol (Gaffney et al., 1993), can often recover the dwarf phenotype to a certain extent (Bowling et al., 1994, Lee et al., 2007, Petersen et al., 2000). However, the recovery is incomplete. My research and others (Beck et al., 2010, Kosetsu et al., 2010) showed that the well characterized negative disease resistant regulator
MPK4 is defective in cell division and cell shape organization in various tissues. Moreover, MPK4 has a direct impact on microtubule bundling (Beck et al., 2010), suggesting that the cell division defect is not a secondary effect of altered disease response. MPK3 and MPK6, as well as their orthologues, are well established biotic and abiotic stress-responsive kinases in Arabidopsis, tobacco, and other plants (Beckers et al., 2009, Ren et al., 2008, Seo et al., 2007, Sharma et al., 2003, Yang et al., 2001). At the same time, their involvement in cell differentiation of the stomatal development, anther lobe differentiation and programmed cell separation suggest a more fundamental role in sustaining the morphology and daily survival of plants. Recently, Stomatal Cytokinesis-Defective1 (SCD1) was reported to negatively regulate the plant innate immunity against bacteria, providing another example of proteins that are genetically independently involved in both cell division and innate immunity (Korasick et al., 2010). However, the mechanism of how a multi-functional protein is involved in both aspects needs to be elucidated.

One important question that affects many phenotype studies is that the same mutations in different ecotypes sometimes give distinct phenotypes (van Zanten et al., 2009). Loss of function of MPK4 in the Columbia background seems to have stronger effect on cytokinesis than in the Landsberg background, even though the insertion type remains the same (Kosetsu et al., 2010). In addition, high temperature and humidity can partially rescue the dwarf phenotype of mpk4 in Ler, but it does not rescue the mpk4-3 mutant (Gao et al., 2008) in Col (data not shown). Nevertheless, my study of the mpk4 allele in Ler background in the context of pollen formation suggests that the proper amount of functional MPK4 is critically important for the meiotic cytokinesis, and the near absence of MPK4 affects this particular process more than the other cytokinesis progression. The observation that normal somatic cell division proceeds in mpk4 (Ler) plants implies that complementary or alternative cellular machinery exists for executing somatic cytokinesis in Ler in the almost complete absence of MPK4. Future studies can help clarify this mechanism.
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