SYSTEMATIC GENETIC INTERACTION ANALYSIS OF MITOGEN-ACTIVATED PROTEIN KINASES IN *ARABIDOPSIS THALIANA*

by

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Systematic Genetic Interaction Analysis of MAP Kinase Pathway Genes in *Arabidopsis Thaliana*

Shih-Heng Su

Under the supervision of Professor Patrick J. Krysan

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The Mitogen-activated protein kinases are highly conserved in all eukaryotic organisms. There are 10 genes encoding MAP Kinase Kinase Kinase Kinases (MAP4K), ca. 80 genes encoding MAP Kinase Kinase Kinases (MAP3K), 10 genes encoding MAP Kinase Kinases (MAP2K), and 20 genes encoding MAP Kinases (MAPK) in Arabidospsis. However, the biological roles have only been identified for a subset of these kinases due to the lack of mutant phenotypes observed when individual genes from these families are inactivated via mutagenesis. In my project, I focused on studying genetic interactions between pairs of mutant loci affecting these MAPK pathway genes as a strategy for uncovering functional associations that are not apparent from studying single-mutants. First, I performed a pilot genetic interaction experiment using two well-known MAPK pathway single-mutants, *mekk1* (a MAP3K) and *mpk4* (a MAPK). These two genes have been previously suggested to function in a pathway that serves to negatively regulated defense responses. Detailed phenotypic

analysis of the single-mutants revealed that these two mutants respond differently to NaCl treatment and their lateral root development. The *mekk1;mpk4* double-mutant caused synthetic lethality. These results suggested that the functions of *MEKK1* and *MPK4* are not limited to a single, linear pathway, and indicated that genetic interaction analysis has the potential to highlight new functions for genes. Based on these initial findings, I expanded my study by performing a large-scale genetic interaction analysis of MAPK pathway related genes. To facilitate this project I developed a high-throughput double-mutant generating pipeline based on a system for growing *Arabidopsis* seedlings in 96-well plates for genotyping. A quantitative root growth-rate assay as well as qualitative phenotyping were used to screen for evidence of genetic interactions in the 289 double-mutants that I produced. In this screen, I identified seven novel genetic interactions. In particular, I found five mutant loci that caused synthetic enhancement of the root growth defects in *mpk4* single-mutant. I also found two mutants that rescue the *mpk4* dwarfism. My results have provided new insights into the functional roles of a number of MAPK pathway genes in *Arabidopsis* and also demonstrate the value of systematic genetic interaction analysis.

ABBREVIATIONS

MAPK, MPK	mitogen-activated protein kinase
MAP2K, MKK	mitogen-activated protein kinase kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MEKK	mitogen-activated protein (MAP) /extra-cellular-signal-related kinase (ERK) kinase kinase
Col-0	Columbia-0 ecotype
L*er*	Landsberg *erecta* ecotype
T-DNA	transfer DNA integrated into the Arabidopsis genome from Agrobacterium tumefaciens
SA	salicylic acid
JA	jasmonic acid
flg22	22-amino acid peptide from flagellin

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Chapter 1

Overview of genetic interaction analysis and Mitogen-Activated Protein Kinase signaling pathways in *Arabidopsis thaliana*

Rapid completion of the genome sequences for several organisms has significantly changed the types of experimental approaches that can be used in the biological sciences field. In addition to traditional forward genetic methods, scientists can now test gene function using reverse genetic strategies (Krysan et al., 1999; Sussman et al., 2000; Sessions et al., 2002). However, the complexities of signaling pathways in organisms are still a challenge for biologists. According to the Arabidopsis genome sequence project, there are ca. 27,500 genes in Arabidopsis (The Arabidopsis Information Resource, 2011), but just 11% of these genes have had functions assigned via experimental evidence (Saito et al., 2008). A likely reason that few gene functions have been elucidated in Arabidopsis is that functional redundancy or overlapping signaling pathways may obscure mutant phenotypes in single-mutant plants (Moore and Purgganan et al., 2005). According to recent studies in yeast, mapping the comprehensive genetic interaction network can help to resolve complex pathways into conceptually and experimentally tractable modules (Tong et al., 2004; Lin et al., 2008). Therefore, studying the genetic interactions between large numbers of gene pairs may

help us to understand more about the functions of single genes and also the functional relationships between genes.

Definition of genetic interaction

The general definition of genetic interaction, also known as Fisher's definition of epistasis, refers to double-mutants which show an unexpected phenotype that cannot easily be explained from the combined phenotypes of the corresponding single-mutants (Boone et al., 2007; Phillips et al., 2008; Gao et al., 2010). Recently, there have been several large-scale genetic interaction analyses performed in a number of model organism, such as *Saccharamyces cerevisiae*, *Caenorhabditis elegans*, and *Escherichia coli* (Tong et al., 2004; Byrne et al., 2007; Typas et al., 2008).

By comparing the phenotypes of double-mutants and single-mutants, genetic interactions can be classified into negative interactions and positive interactions (Dixon et al., 2009). Figure 1 provides an overview of the different types of genetic interaction that have been described. The definition of a negative genetic interaction, also known as synthetic enhancement, refers to a double-mutant whose phenotype is more severe than expected. For example, synthetic lethality is the most extreme case

of negative genetic interaction. Positive interaction refers to a double-mutant whose phenotype is less severe than expected. This kind of interaction is also called an alleviating interaction or synthetic rescue (Dixon et al., 2009; Mani et al., 2008).

The gene pairs found to display genetic interaction are not necessarily partners in a protein-protein interaction or a protein complex (Ma et al., 2008). There are several other mechanisms that have the potential to cause genetic interaction. One is that the pair of genes belong to parallel pathways that regulate the same essential biological process. Although the single-mutants may not alter the essential biological processes, the double-mutants partially or fully block the pathways (Boone et al., 2007). A second potential mechanism is one in which two genes belong to the same signaling pathway but show functional redundancy. In this model, the two genes can substitute for each other's function and single-mutants will not show an abnormal phenotype, while the double-mutant that carries neither gene has an altered phenotype.

Systematic genetic interaction analysis in yeast

Saccharomyces cerevisiae, or yeast, is a unicellular eukaryotic organism. Based on the genome sequence from yeast, there are ca. 6,000 open reading frames that are

predicted to encode proteins. However, just 18% of yeast genes are essential for the viability of yeast cells (Ooi et al., 2006). Methodologies for large-scale genetic interaction analysis have been well established in *S. cerevisiae*. The most well-known method is called synthetic genetic array (SGA) (Tong et al., 2001). This method begins with a *MATα* yeast strain that carries a single mutation, called a query mutation, linked to a dominant selectable marker. Mating is then performed with an ordered array of *MATa* deletion mutants, a library of target mutants, in which single genes are disrupted by the insertion of a second selectable marker. Then, antibiotics are used to select double-mutants that carry one query mutation and one target mutation. The presence of genetic interaction is determined by scoring the fitness of these double-mutants and comparing it with the fitness of each single-mutant (Boone et al., 2007).

The first large-scale genetic interaction experiment in yeast involved 132 synthetic genetic array screens. Each of the 132 chosen query mutants was screened against a library of 4,700 non-essential mutants (Tong et al., 2004). In this study, the authors observed an average rate of 34 interactions per query gene based on the measurement of synthetic lethality. The rate of genetic interaction reported in this experiment was ca. 0.7%. In this study 6,119 genetic interactions were found

between just 1,440 of the 4832 genes. This information implies that genetic interaction doesn't occur randomly and that the choice of input genes could have a major effect on the ability to detect genetic interactions, and the rate of that detection. The second large-scale analysis in yeast using a synthetic genetic array screen was reported in 2010, in which 1712 query mutants were used for screening, generating ca. 5.4 million double-mutants. The major difference in this study was the scoring system for the phenotypic assay: the authors used colony size as a quantitative index rather than simply lethality in order to determine the fitness of double-mutants. In this second study, the authors found an interaction rate of ca. 3% (Costanzo et al., 2010). These results provide the evidence that using a quantitative index can increase the identification of interactions.

Large-scale genetic interaction analysis in *Caenorhabditis elegans*

Following the successful analysis of genetic interactions in yeast, scientists wanted to apply a similar systematic approach to a multi-cellular animal system. In 2006, the first large-scale analysis of genetic interaction in an animal was reported using *Caenorhabditis elegans* (Lehner et al., 2006). There are two major differences in this

study compared to yeast. First, since there is no comprehensive collection of single-mutants in *C. elegans*, the RNAi method was used to produce silenced strains of *C. elegans*. Second, instead of using all the genes from the genome as the screening library, the authors selected a set of 1,750 genes for their study that were predicated by sequence homology to function in signal transduction, transcription regulation and chromatin remodeling. Genetic interactions were identified by observing extreme phenotypes such as embryo lethality, brood size defects, growth defects, and larval lethality (Lehner et al., 2006). There were ca. 350 genetic interactions observed from ca. 65,000 combinations, giving a rate of genetic interaction of 0.5%.

Another group developed a somewhat different approach, which they called systematic genetic interaction analysis (SGI) (Byrne et al., 2007). The SGI approach also relies on RNAi to generate double-mutant lines in *C. elegans*. However, genetic interactions were determined based on a scoring system which analyzed the double-mutant phenotypes quantitatively instead of relying on qualitative traits. Eleven query genes were used to search against 858 signaling pathway related target genes, and 1,246 interactions were observed (Byrne et al., 2007). The rate of detecting genetic interaction in this study was 13%. Comparing these two *C. elegans* studies, in combination with the studies in yeast, implies that quantitative phenotype

measurement can highly increase the chance of observing evidence of genetic interaction.

Application of genetic interaction networks

In order to organize the large amount of data produced by large-scale genetic interaction analysis, yeast scientists utilized a genetic interaction network map to present their results (Tong et al., 2004; Davierwala et al., 2005; Boone et al., 2007; Dixon et al., 2009; Costanzo, et al., 2010). Based on this map, it was observed that genetic interactions were highly biased toward genes that have share functions or are involved in similar biological processes (Tong et al., 2004). In other words, a genetic interaction network has the potential to predict functional pathways, predict essential genes that are critical to viability, or even understand possible relationships between pathways that are involved in different biological processes.

Introduction to the MAPK cascade

Mitogen-activated protein kinase (MAPK) modules are signal transduction cascades that are highly conserved throughout evolution. There are three classes of

kinases involved in these cascades: MAP Kinase Kinase Kinases (MAP3K), MAP Kinase Kinases (MAP2K), and MAP Kinases (MPK) (Ferrell, 1996). Some pathways also include a MAP Kinase Kinase Kinase Kinase (MAP4K) acting upstream of the MAP3K (Wu et al., 1995). In a typical MAPK signaling pathway, a MAP3K is activated by upstream proteins, such as a receptor-like kinase or a MAP4K. MAP3Ks are serine/threonine kinases that recognize and phosphorylate the S/T-X_{3-5}-S/T motif of MAP2Ks. The activated MAP2Ks will recognize and phosphorylate the threonine and tyrosine motif (T-X-Y) of MAPKs. MAPKs typically phosphorylate downstream transcription factors that regulate the expression of genes in response to the upstream signal, in addition to phosphorylating other substrate proteins (Widmann et al., 1999).

MAPK pathways in yeast

In the yeast *Saccharomyces cerevisiae*, there is only one *MAP4K,* four *MAP3K*s, four *MAP2K*s, and six *MAPK*s and (Widmann et al., 1999; Qi and Elion, 2005). The five major MAPK signaling pathways in yeast are well-characterized: the pheromone response pathway, the filamentous growth pathway, the high osmolarity pathway, the cell wall integrity pathway, and the spore wall assembly pathway (Chen and Thorner,

2007). In MAPK-mediated pathways, an important class of proteins called scaffold proteins play a key role in ensuring that components of the MAPK cascades interact with the proper upstream and downstream kinases (Chen and Thorner, 2007). Although they are important, there is no apparent sequence relatedness between different scaffold proteins. In some yeast pathways, a kinase can itself act as a scaffold protein, such as the *MAP2K* gene *Pbs2* (Zarrinpar et al., 2004).

MAPK pathways in Arabidopsis

In *Arabidopsis thaliana,* MAP kinase cascades have been shown to play important roles in signaling pathways related to growth and development as well as responses to environmental stress stemming from both biotic and abiotic factors (Ichimura et al., 2000; Droillard et al., 2004 ; Teige et al., 2004; Hadiarto et al., 2006; Gudesblat et al., 2007; Colcombet and Hirt, 2008). Based on the Arabidopsis genome annotation from the TAIR genome database version 10, there are ca. 27,416 predicted genes in the Arabidopsis genome (The Arabiodpsis Information Resource, 2011). There are 10 *MAP4K*s, 80 *MAP3*Ks, 10 *MAP2K*s and 20 *MAPK*s encoded in the Arabidopsis

genome (Huala et al., 2001). However, very few gene functions or obvious abnormal phenotypes have been associated with MAP kinase pathway genes.

Perhaps the best studied MAPK signaling pathways in Arabidopsis are those related to defense response. There are at least two complete MAPK modules that have been identified that are involved in pathogen response signaling. First, a pathway involving the genes *MEKK1* (MAP3K), *MKK4/MKK5* (MAP2K), and *MPK3/MPK6* (MAPK) activates an ethylene synthesis- and jasmonate (JA)-related pathogen response in Arabidopsis (Asai et al., 2002). This study was conducted using a protoplast system. Second, a pathway involving *MEKK1* (MAP3K), *MKK1/MKK2* (MAP2K), and *MPK4* (MAPK) negatively regulates the salicylic acid -mediated systemic acquire resistance response (Petersen et al., 2000; Teige et al., 2004). There are several additional reports showing that individual MAPK pathway genes are involved in various pathogen defense pathway. For example, the MAP2K *MKK3* expression is induced by pathogen treatment, and the active form of the MKK3 protein positively regulates expression of *Pathogen-Related (PR)* genes (Doczi et al., 2007). Plants over-expressing the MAP2K gene *MKK7* induce plant basal defenses and also show greater resistance to pathogen infection (Dai et al., 2005; Zhang et al., 2007).

Another important biological process in which MAPK cascades are involved is phytohormone biosynthesis and signaling pathways. For example, the enzymatic activity of the ACS6 protein, which encodes an ACC synthase involved in ethylene biosynthesis, can be regulated through phosphorylation by the MAPK proteins MPK3 and MPK6 (Liu & Zhang 2004; Han et al. 2010). In addition, a signaling cascade involving the MAP2K MKK9 and the MAPKs MPK3/6 has been suggested to play an important role in ethylene signal transduction (Yoo et al., 2008). A jasmonic acid signaling pathway has been shown to be affected by the MAP2K *MKK3* and the MAPK *MPK6* (Takahashi et al., 2007), while overexpression of the MAP2K *MKK7* results in deficiency in auxin transport (Zhang et al., 2007). Finally, abscisic acid-mediated stomatal opening was found to be regulated in part by the functionally redundant actions of the MAPKs *MPK9* and *MPK12* (Jammes et al., 2009).

MAPK signaling cascades are also involved in pathways that control growth and development in Arabidopsis. For example, the MAP3K gene *YODA* plays a critical role controlling embryo development. The *yoda* single-mutant plant causes reduced cell elongation in the embryo, leading to the failure of the zygote to differentiate its suspensor cell (Lukowitz et al, 2004). A pathway composed of *Yoda,* the MAP2Ks

MKK4/MKK5, and the MAPKs *MPK3/MPK6* has been shown to control stomatal development and patterning in Arabidopsis (Wang et al., 2007).

Cell division has also been shown to be affected by MAPK pathways. A module composed of the MAP3Ks *ANP2/ANP3,* the MAP2K *MKK6,* and the MAPK *MPK4* appears to be involved in the regulation of cytokinesis as evidenced by the observation that the *anp2;anp3* double-mutant, the *mkk6* single-mutant, and the *mpk4* single-mutant all show similar defects in cytokinesis (Krysan et al., 2002; Takahashi et al., 2010; Kosetsu et al., 2010). Additionally, abiotic stresses also can induce MAPK signaling cascades, such as cold, salt, drought, wounding, osmotic stress, and ozone (Droillard et al., 2004; Ahlfors et al., 2004; Teige et al., 2004; Gudesblat et al., 2007). Table 1 gives an overview of the current knowledge regarding MAPK signaling pathways in Arabidopsis.

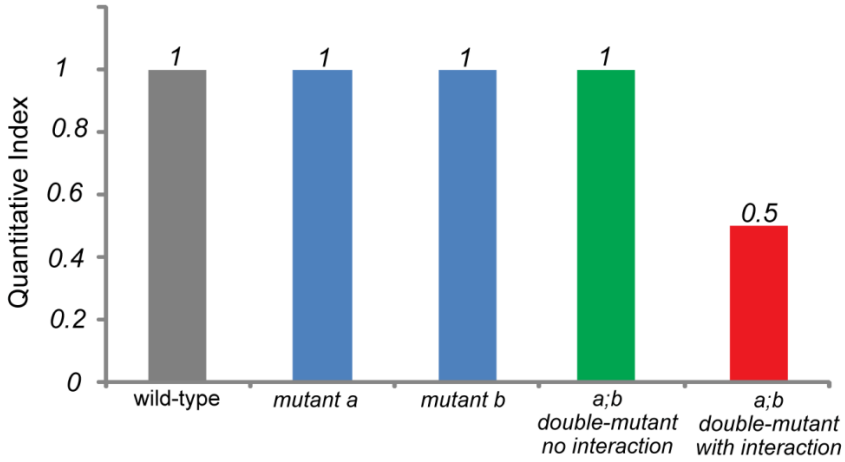
Genetic interactions of MAPK cascade in Arabidopsis

Although no large-scale genetic interaction analyses have been done in Arabidopsis, there are some individual reports showing genetic interactions between MAPK pathway genes. Most of the genetic interactions that have been identified are

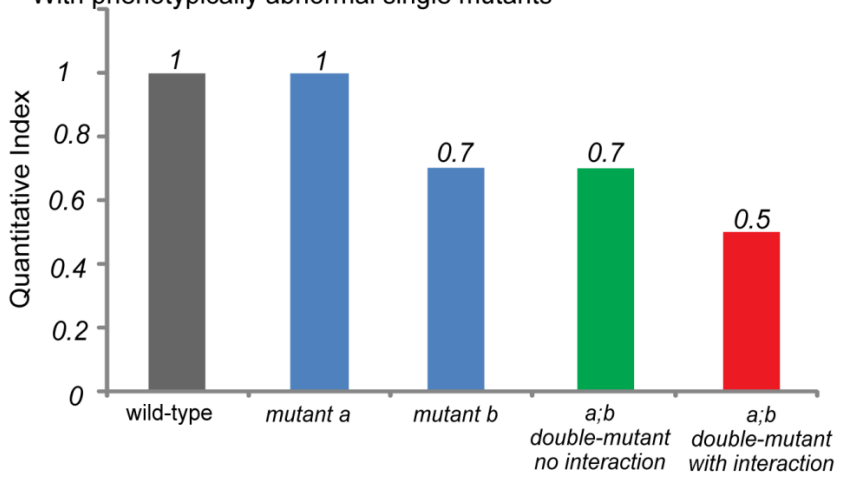
between pairs of genes within the same gene family. For example, single-mutants of *mpk3* and *mpk6* show a largely wild-type phenotype. However, the *mpk3;mpk6* double-mutant combination results in synthetic embryo lethality (Wang et al., 2007). The *mpk4* single-mutant has a strongly dwarfed phenotype, but *mpk4;mpk11* double-mutants show a more severe dwarfism than *mpk4* alone (Kosetsu et al., 2010). *mpk9;mpk12* double-mutants are insensitive to ABA treatment, while each of the corresponding single-mutants has normal ABA responses (Jammes et al., 2009). *mkk1*;*mkk2* double-mutant plants show a strong dwarf phenotype which is not observed with either *mkk1* or *mkk2* single-mutant plants (Qiu et al., 2008). The *map3kε1*;*map3kε2* double-mutant combination causes pollen lethality (Chaiwongsar et al., 2006), though both single-mutants have normal pollen. *anp1;anp3* and *anp2;anp3* double-mutant plants show defects in cell division and growth (Krysan et al., 2002). All of these examples involve gene pairs that are members of the same gene family, and the concept of functional redundancy is typically used to explain the appearance of a mutant phenotype in the double-mutant that is not observed in either of the corresponding single-mutants. For my project I was interested not only in this type of functional redundancy within gene families, but also in identifying genetic interactions that crossed gene family boundaries. This type of interaction could not be explained

by simple functional redundancy, and could potentially offer novel insights into signaling pathways. In this thesis I will describe the systematic genetic interaction studies I completed in Arabidopsis during my doctoral work.

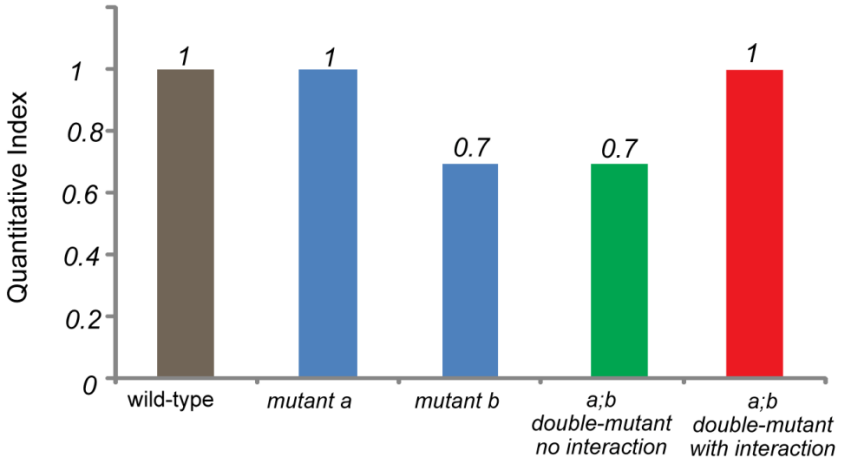
(A) Negative interaction -
With phenotypically wild-type single mutants



(B) Negative interaction -
With phenotypically abnormal single mutants



(C) Positive interaction - Asymmetric



(D) Positive interaction - symmetric

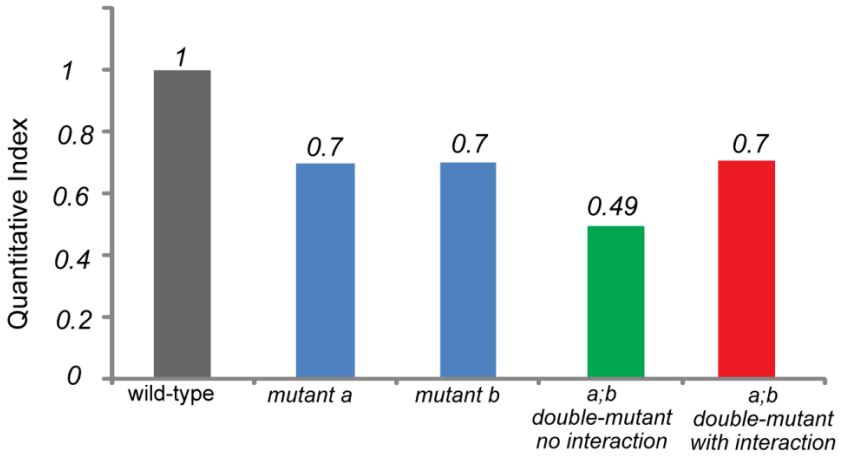


Fig. 1. Hypothetical examples of genetic interaction (Modified from Dixon et al., 2009). Gray indicates the quantitative phenotype of wild-type. Blue indicates each of the two single-mutants. Green indicates the expected phenotype when two mutants show no evidence of genetic interactions. Red indicates the expected phenotype when two mutants display genetic interaction. (A) Negative interaction when the two single-mutants a and b display the same phenotype as wild-type. (B) Negative interaction when one of the single mutants has a phenotype that is significantly different from wild-type. (C) Asymmetric positive interactions. (D) Symmetric positive interactions.

Table 1. Known MAPK cascade gene function in Arabidopsis

Stimuli	*Genes or Modules*	*Response*	*Reference*
Pathogen defense			
	MEKK1-MKK4/5-MPK3/6	*Pathways are activated by pathogen treatment*	*Asai et al., 2002*
	MEKK1-MKK1/2-MPK4	*mekk1, mkk1;mkk2, mpk4 show dwarfism caused by activated SA mediated pathogen response*	*Teige et al., 2004*
	ANP1-MKK4/5--MPK3/6	*Regulate Auxin and H_2O_2 mediated gene expression*	*Kovtun et al., 2000*
	MEKK1- ? -MPK4	*Pathways are activated by pathogen treatment*	*Nakagami et al., 2006*
flg22	*? –MKK3-MPK6*	*Regulate JA-induced gene expression*	*Takahashi et al., 2007*
$H_2$02	*? -MKK3-MPK7*	*Positively regulate Pathogen-Related genes*	*Doczi et al., 2007*
	?-MKK7-?	*Overexpression of MKK7 activates basal defense responses and SAR*	*Zhang et al., 2007*
Phytohormone			
ET	*?-MKK9-MPK3/6*	*Activates EIN3*	*Yoo et al., 2008*
	?-?-MPK6	*Activates ACS to up-regulate ethylene biosynthesis*	*Liu and Zhang, 2004*
Auxin	*?-MKK7-?*	*Overexpression of MKK7 causes auxin transportation deficiency*	*Dai et al., 2006*
	?-?-MPK12	*mpk12 is insensitive auxin*	*Lee et al., 2008*
ABA	*? –MKK1-MPK3/6*	*Regulates seed germination*	*Xing et al., 2009*
	?-?-MPK9/MPK12	*Regulates ABA mediated stomatal opening*	*Jammes et al., 2009*
Abiotic stress			
Cold	*MEKK1-MKK2-MPK4/6*		*Teige et al., 2004* *Ichimura et al., 2000*
Ozone	*?-?-MPK3/6*		*Ahifors et al., 2004* *Ichimura et al., 2000*
Salt	*MEKK1-MKK2-MPK4/6*		*Teige et al., 2004* *Ichimura et al., 2000*

Table 1. Known MAPK cascade gene function in Arabidopsis. (Continued)

Stimuli	*Genes or Modules*	*Response*	*Reference*
Abiotic stress			
Drought	*? –MKK1-MPK4*		*Gudesblat et al., 2007*
Wounding	*MEKK1-MKK1-6*		*Hadiarto et al., 2006*
			Ichimura et al., 2000
Osmotic	*?- ?- MPK3/4/6*		*Droillard et al., 2004*
Development			
	ANP1/ANP2/ANP3-MKK6-MPK4	*Mutants show cytokinesis defects*	*Krysan et al., 2002;*
	YODA-?-MPK6	*Regulates embry differentiation*	*Lukowtiz et al., 2004*
			Bush and Krysan, 2007
	YODA- MKK4/5-MPK3/6	*Regulates stomatal patterning*	*Wang et al., 2007*
	?-MKK4/5-MPK3/6	*Regulates floral organ abscission*	*Cho et al., 2008*
	YODA-MKK7/MKK9-?	*Regulates stomata development*	*Lampard et la., 2009*
	?-?-MPK18	*Microtubule stability*	*Walia et al., 2009*
Other			
	?-MKK6-MPK13	MKK6 *may activate* MPK13	*Melikant et al., 2004*

LITERATURE CITED

Ahlfors, R., Macioszek, V., Rudd, J., Brosche, M., Schlichting, R., Scheel, D., Kangasjarvi, J. (2004) Stress hormone-independent activation and nuclear translocation of mitogen-activated protein kinases in Arabidopsis thaliana during ozone exposure. Plant J 40: 512-522.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature 415: 977-983.

Boone, C., Bussey, H., and Andrews, B.J. (2007) Exploring genetic interactions and networks with yeast. Nat. Rev. Genetics 8:437-449.

Byrne, A.B., Weirauch, M.T., Wong, V., Koeva, M., Dixon, S.J., Stuart, J.M., and Roy, P.J. (2007) A global analysis of genetic interactions in Caenorhabditis elegans. J Biol. 6:8.

Chaiwongsar, S., Otegui, M.S., Jester, P.J., Monson, S.S., and Krysan, P.J. (2006) The protein kinase genes MAP3Kε1 and MAP3Kε2 are required for pollen viability in Arabidopsis thaliana. Plant J. 48:193-205.

Chen, R.E., and J. Thorner. (2007) Function and regulation in MAPK signaling pathways. Biochim Biophys Acta. 1773:1311-1340.

Colcombet, J., and Hirt, H. (2008) Arabidopsis MAPKs: a complex signaling network involved in multiple biological processes. Biochem. J. 413:217-226.

Costanzo, M., Baryshnikova, A., Myers, C.L., Andrews, B., and Boone, C. (2011) Charting the genetic interaction map of cell. Current opinion in Biotechnology 22:66-74.

Dai, Y., Wang, H., Li, B., Huang, J., Liu, X., Zhou, Y., Mou, Z., and Li, J. (2006) Increased expression of MAP KINASE KINASE7 causes deficiency in polar auxin transport and leads to plant architectural abnormality in Arabidopsis. Plant Cell 18: 308-320.

Davierwala, A.P., Haynes, J., Li, Z., Brost, R.L., Robinson, M.D., Yu, L., Mnaimneh, S., Ding, H., Zhu, H., Chen, Y., Cheng, X., Brown, G.W., Boone, C., Andrews, B.J., and Hughes, T.R. (2005) The synthetic genetic interaction spectrum of essential genes. Nature genetics 37:1147-1152.

Dixon, S.J., Costanzo, M., Baryshnikova, A., Andrews, B., and Boone, C. (2009) Systematic mapping of genetic interaction networks. Annu. Rev. Genet. 43:601-625.

Doczi, R., Brader, G., Pettko-Szandtner, A., Rajh, I., Djamei, A., Pitzschke, A., Teige, M., and Hirt, H. (2007) The Arabidopsis mitogen-activated protein kinase kinase MKK3 is upstream of group C mitogen-activated protein kinases and participates in pathogen signaling. Plant Cell 19: 3266-3279.

Droillard, M., Boudsocq, M., Barbier-Brygoo, H., and Laurière, C. (2004) Different protein kinase families are activeated by osmotic stresses in Arabidopsis thaliana cell suspensions. Involvement of the MAP kinases At MPK3 and At MPK6. FEBS Lett. 527:43-50

Ferrell, J.E. Jr. 1996. MAP kinases in mitogenesis and development. Curr Top Dev Biol. 33:1-60.

Gao, H., Granka J.M., and Feldman, M.W. 2010. On the classification of epistatic interactions. Genetics 184:827-837.

Gudesblat, G.E., Iusem, N.D., and Morris, P.C. (2007) Guard cell-specific inhibition of Arabidopsis MPK3 expression causes abnormal stomatal responses to abscisic acid and hydrogen peroxide. New Phytol. 173: 713-721.

Han, L., Li, G-J., Yang, K-Y., Mao, G., Wang, R., Liu, Y., and Zhang, S. (2010) Mitoge-activated protein kinase 3 and 6 regulate Botrytis cinerea-induced production in Arabidopsis. Plant J. 64: 114-127.

Huala, E., Dickerman, A.W., Garcia-Hernandez, M., Weems, D., Reiser, L., LaFond, F., Hanley, D., Kiphart, D., Zhuang, M., Huang, W., Mueller, L.A., Bhattacharyya, D., Bhaya, D., Sobral, B.W., Beavis, W., Meinke, D.W., Town, C.D., Somerville, C., and Rhee, S.Y. (2001) The Arabidopsis Information Resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. Nucleic Acids Res. 29:102-5.

Jammes, F., Song, C., Shin, D., Munemasa, S., Takeda, K., Gu, D., Cho, D., Lee, S., Giordo, R., Sritubtim, S., Leonhardt, N., Ellis, B.E., Murata, Y., and Kwak, J.M. (2009) MAP kinases MPK9 and MPK12 are preferentially expressed in guard cells and positively regulate ROSmediated ABA signaling. Proc Natl Acad Sci USA 106: 20520-20525.

Kosetsu, K.K., Matsunage, S., Nakagami, H., Colcombet, J., Sasabe, M., Soyano, T., Takahashi, Y., and Machida, Y. (2010) The MAP kinase MPK4 is required for Cytokinesis in Arabidopsis thaliana. Plant Cell 22: 3778-3790.

Krysan, P.J., Young, J.C., and Sussman, M.R. (1999) T-DNA as an insertinal Mutagen in Arabidopsis. Plant Cell 11:2283-2290.

Krysan, P.J., Jester, P.J., Gottwald, J.R., and Sussman, M.R. (2002) An Arabidopsis mitogen-activated protein kinase kinase kinase gene family encodes essential positive regulators of cytokinesis. Plant Cell 14:1109-1120.

Lehner, B., Crombie, C., Tischler, J., Fortunato, A., and Fraser, A.G. (2006) Systematic mapping of genetic interactions in Caenorhabditis elegans identifies common modifiers of diverse signaling pathways. Nat. Genet. 38:896-903.

Lin et al., 2008. A comprehensive synthetic genetic interaction network governing yeast histone acetylation and deacetylation. Genes & Development, 22:2062-2074.

Lin, Y.Y., Qi, Y., Lu, J.Y., Pan, X., Yuan, D.S., Zhao, Y., Bader, J.S., and Boeke, J.D. (2004) Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. Plant Cell 16, 3386-3399.

Lukowitz, W., Roeder, A., Parmenter, D., and Somerville, C. (2004) A MAPKK Kinase Gene Regulates Extra-Embryonic Cell Fate in Arabidopsis. Cell 116: 109-119

Ma, X., Tarone, A.M., Li, W. (2008) Mapping genetically compensatory pathways from synthetic lethal interactions in yeast. PLoS ONE, 3:e1922.

Mani, R., St Onge, R.P., Hartman, J.L. 4th, Giaever, G., and Roth, F.P. (2008) Defining genetic interaction. Proc Natl Acad Sci USA 105:3461-3466.

Moore, R.C., and Purugganan, M.D. (2005) The evolutionary dynamics of plant duplicate genes. Curr Opin Plant Biol. 8: 122-128.

Ooi, S.L, Pan, X., Peyser, B.D., Ye, P., Meluh, P.B., Yuan, D.S., Irizarry, R.A., Bader, J.S., Spencer, F.A., and Boeke, J.D. (2006) Global synthetic-lethality analysis and yeast functional profiling. Trends in Genetics 22: 56-63.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J. (2000) Arabidopsis MAP Kinase 4 negatively regulates systemic acquired resistance. Cell 103: 1111-1120.

Phillips, P.C. (2008) Epistasis-the essential role of gene interactions in the structure and evolution of genetic systems. Nat Rev Genet. 9:855-867.

Qi, M., and Elion, E.A. (2005) MAP kinase pathways. J Cell Sci. 118:3569-3572.

Qiu, J.L., Zhou, L., Yun, B.W., Nielsen, H.B., Fiil, B.K., Petersen, K., Mackinlay, J., Loake, G.J., Mundy, J., Morris, P.C. (2008b) Arabidopsis mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. Plant Physiol 148: 212-222.

Saito, K., Hirai, M.Y., and Yonekura-Sakakibara, K. (2008) Decoding genes with coexpression networks and metabolomics-‘majority report by precogs’. Trends Plant Sci. 13:36-43.

Sessions, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J., Ko, C., Clarke, J.D., Cotton, D., Bullis, D., Snell, J., Miguel, T.,

Hutchison, D., Kimmerly, B., Mitzel, T., Katagiri, F.,Glazebrook, J., Law, M., and Goff, S.A. (2002) A high-throughput Arabidopsis reverse genetics system. Plant Cell 14:2985-2994

Sussman, M.R., Amasino, R.M., Young, J.C., Krysan, P.J., and Austin-Phillips, S. (2000) The Arabidopsis knockout facility at the University of Wisconsin-Madison. Plant Physiol. 124:1465-1467.

Takahashi, F., Yoshida, R., Ichimura, K., Mizoguchi, T., Seo, S., Yonezawa, M., Maruyama, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2007) The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. Plant Cell 19: 805-818.

Takahashi, Y., Soyano, T., Kosetsu, K., Sasabe, M., and Machida, Y. (2010) HINKEL kinesin, ANP MAPKKKs and MKK6/ANQ MAPKK, which phosphorylates and activates MPK4 MAPK, constitute a pathway that is required for cytokinesis in Arabidopsis thaliana. Plant Cell Physiol. 51: 1766-1776.

Teige, M., Scheikl, E., Eulgem, T., Dóczi, R., Ichimura, K., Shinozaki, K., Dangl, J.L., Hirt, H. (2004) The MKK2 Pathway Mediates Cold and Salt Stress Signaling in Arabidopsis. Molecular Cell 15: 141-152.

The Arabidopsis Genome initiative. (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature 408:796-815.

Tong, A.H., Evangelista, M., Parsons, A.B., Xu, H., Bader, G.D., Pagé, N., Robinson, M., Raghibizadeh, S., Hogue, C.W., Bussey, H., Andrews, B., Tyers, M., and Boone, C. (2001) Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science 294:2364-2368.

Tong, A.H., Lesage, G., Bader, G.D., Ding, H., Xu, H., Xin, X., Young, J., Berriz, G.F., Brost, R.L., Chang, M., Chen, Y., Cheng, X., Chua, G., Friesen, H., Goldberg, D.S., Haynes, J., Humphries, C., He, G., Hussein, S., Ke L, Krogan, N., Li, Z., Levinson, J.N., Lu, H., Ménard, P., Munyana C, Parsons, A.B., Ryan, O., Tonikian, R., Roberts, T., Sdicu, .M., Shapiro, J., Sheikh, B., Suter, B., Wong, S.L., Zhang, L.V., Zhu, H., Burd, C.G., Munro, S., Sander, C., Rine, J., Greenblatt, J., Peter, M., Bretscher, A., Bell, G., Roth, F.P., Brown, G.W., Andrews, B., Bussey, H., and Boone, C. (2004) Global mapping of the yeast genetic interaction network. Science 303:808-813.

Typas, A., Nichols, R.J., Siegele, D.A., Shales, M., Collins, S.R., Lim, B., Braberg, H., Yamamoto, N., Takeuchi, R., Wanner, B.L., Mori, H., Weissman, J.S., Krogan, N.J., and Gross, C.A. (2008) High-throughput, quantitative analyses of genetic interactions in E. coli. Nature Methods 5:781-787.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C., Zhang, S. (2007) Stomatal Development and Patterning Are Regulated by Environmentally Responsive Mitogen-Activated Protein Kinases in Arabidopsis. Plant Cell 19: 63-73.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999) Mitogen-Activated Protein Kinase: Conservation of a Three-Kinase Module From Yeast to Human. Physiol Rev. 79: 143-180

Wu, C., Whiteway, M., Thomas, D.Y., and Leberer, E. (1995) Molecular characterization of Ste20p, a potential mitogen-activated protein or extracellular signal-regulated kinase kinase (MEK) kinase kinase from Saccharomyces cerevisiae. J Biol Chem. 270:15984-15992.

Yoo, S.D., Cho, Y.H., Tena, G., Xiong, Y., and Sheen, J. (2008) Dual control of nuclear EIN3 by bifurcate MAPK cascades in C2H4 signalling. Nature 451:789-95

Zarrinpar, A., Bhattacharyya, R.P., Nittler, M.P., and Lim, W.A. (2004) Sho1 and Pbs2 act as coscaffolds linking components in the yeast high osmolarity MAP kinase pathway. Mol. Cell. 14:825-832.

Zhang, X., Dai, Y., Xiong, Y., Defraia, C., Li, J., Dong, X., and Mou, Z. (2007) Overexpression of Arabidopsis MAP kinase kinase 7 leads to activation of plant basal and systemic acquired resistance. Plant J. 52: 1066-1079.

Chapter 2

Genetic interaction and phenotypic analysis of the Arabidopsis MAP kinase pathway mutations *mekk1* and *mpk4* suggests signaling pathway complexity

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ABSTRACT

It has been shown that the Arabidopsis MEK kinase *MEKK1* acts upstream of the MAP kinase *MPK4* to negatively regulate salicylic acid-dependent defense-response pathways. Here, we report that the *mekk1;mpk4* double-mutant combination causes seedling lethality. In addition, we demonstrate that *mekk1* and *mpk4* single-mutant plants have significantly different phenotypes. *mekk1* plants are defective for lateral root formation, while *mpk4* plants are not. In addition, treatment with elevated levels of sodium chloride improves the growth of *mekk1* plants, while it inhibits the growth of *mpk4* plants. Our results suggest that *MEKK1* and *MPK4* functions are not limited to a single, linear signaling pathway. Instead there appears to be more complexity to the signaling pathways in which these two proteins function.

INTRODUCTION

Mitogen activated protein kinase (MAPK) signaling cascades have been implicated in the regulation of many aspects of plant growth and development. (Jonak et al., 2002; Mishra et al., 2006; Tena et al., 2001) It has recently been demonstrated that the Arabidopsis MAP kinase kinase kinase *MEKK1* acts upstream of the MAP kinase *MPK4*, and that these proteins act in a signaling pathway that negatively regulates the activation of defense-response genes and reactive oxygen species production. (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007) Null mutants for *mpk4* and *mekk1* have similar phenotypes that are characterized by dwarfism, true leaves that are dark-green and curled, constitutive H_2O_2 accumulation in leaves, and constitutive expression of defense response genes. (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Brodersen et al., 2006; Petersen et al., 2000) Both *mpk4* and *mekk1* mutants grow poorly at normal growth temperatures but are able to survive for several weeks in soil. (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007) An additional characteristic of *mekk1* plants that has been reported is that the dwarf phenotype can be largely rescued by growing the plants at elevated temperatures in the range of 28–32^{0}C. (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007) This type of high-temperature rescue has also been

reported for other mutants that cause dwarfism due to constitutive expression of defense-response genes. (Ichimura et al., 2006)

Based on several recent publications, the current model for *MEKK1* and *MPK4* function is that a MAP kinase cascade composed of *MEKK1*, *MKK1*, and *MPK4* exists in Arabidopsis that serves as a negative regulator of pathogen response pathways. (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007; Meszaros et al., 2006) This cascade acts downstream of the flagellin receptor *FLS2*, which detects the presence of pathogen-associated molecular patterns (PAMPS) such as the flagellin peptide flg22. (Chinchilla et al., 2006) MKK1 is a MAP kinase kinase that has been shown to be required for the full activation of MPK4 in response to flg22 treatment. (Meszaros et al., 2006)

The spatial expression patterns of *MEKK1* and *MPK4* in leaf tissue have been previously characterized through the use of transcriptional fusions between the native promoter of each gene and the *GUS* reporter gene. (Ichimura et al., 2006; Petersen et al., 2000) For both genes the strongest expression was observed in guard cells and vascular tissue. It was also reported that *MEKK1* was more strongly expressed in emerging true leaves when compared to mature leaves. (Ichimura et al., 2006) Root tissue was not examined in either of these studies. The response of Arabidopsis to

flg22 treatment is not limited to the *MEKK1/MKK1/MPK4* cascade described above. It has also been shown that the MAP kinases *MPK3* and *MPK6* become activated in response to flg22 treatment. (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007; Meszaros et al., 2006; Droillard et al., 2004) These results indicate that the structure and identity of the MAP kinase cascades that react to a given stimulus in plants are likely to be characterized by complexity. Because the Arabidopsis genome encodes 20 MAPKs, 10 MAP2Ks, and at least 60 MAP3Ks, the potential for complexity is high. (MAPK Group, 2002) In the present study, we have performed double-mutant analysis of the mekk1 and mpk4 mutations in order to better understand the genetic interactions between these loci. If *MEKK1* and *MPK4* act in a single, linear signaling pathway then one would expect double-mutant plants to have a phenotype that is identical to that of *mpk4* mutants, since it has been demonstrated that *MEKK1* acts upstream of *MPK4*. Here, we report results suggesting that signaling through *MEKK1* and *MPK4* is more complex than this simple linear model would predict.

RESULTS

The mekk1 and mpk4 dwarf phenotypes are rescued by growth at high temperature

It has been demonstrated that the dwarf phenotype of mekk1 single-mutant plants is largely rescued by growth at elevated temperatures in the range of 28–32^{0}C. (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007) It is also known that *mpk4* single-mutant plants have a dwarf phenotype that is very similar to that of *mekk1* mutants (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Brodersen et al., 2006; Petersen et al., 2000). Because *MPK4* has been shown to act downstream of *MEKK1* in the regulation of defense-response pathways (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007), we were interested in determining if the *mpk4* dwarf phenotype could also be rescued by high-temperature growth. Seeds of *mekk1-1* in the Columbia background, *mpk4* in the Landsberg background, wild-type Columbia, and wild-type Landsberg were sown on soil and placed under constant light at 22^{0}C for five days. During this time period the seedlings of all the genotypes germinated. The dwarf phenotypes of the *mekk1* and *mpk4* mutants are not apparent at this early stage of development. The plants were then transferred to 30^{0}C and grown continuously at this temperature for an additional 10 days. As seen in figure 1, the *mpk4* dwarf phenotype is rescued by growth at elevated temperatures, indicating that this characteristic is shared by both the *mekk1* and *mpk4* mutants.

Sodium chloride treatment differentially affects mekk1 and mpk4 mutant plants

Previous reports have indicated that *MEKK1* transcription is up-regulated when plants are subjected to osmotic and/or salt stress. (Mizoguchi et al., 1996) We therefore tested the response of *mekk1-1* seedlings to osmotic stress by supplementing their growth media with various concentrations of mannitol, sucrose and glucose (see material and methods). The response of *mekk1-1* seedlings to these treatments was similar to that displayed by wild-type (data not shown). Media containing elevated levels of sodium chloride, however, were found to improve the growth of *mekk1-1* plants (Fig. 2A–C). The true leaves formed by *mekk1* plants grown on basal media are dark green and poorly expanded. When grown in the presence of 75 mM sodium chloride, however, the true leaves of *mekk1* plants display substantially improved expansion (Fig. 2B). For comparison, the growth of wild-type plants is progressively inhibited by increasing sodium chloride levels (Fig. 2A,C). The fresh-weight of *mekk1-1* plants grown in the presence of 75mM sodium chloride is ca. threefold greater than that of *mekk1-1* plants grown on basal media (Fig. 2C). In addition, the average fresh weights of *mekk1-1* plants grown on 50 mM, 75 mM, and 100 mM sodium chloride were all significantly greater than that of *mekk1-1* plants grown on basal media (t-test, $P< 0.01$). By contrast, growth in the presence of 75 mM sodium chloride reduces the fresh weight

of wild-type Columbia plants ca. fourfold when compared to wild-type grown on basal media (Fig. 2C). Because mannitol, sucrose, and glucose treatments were not able to rescue the *mekk1-1* phenotype, it appears that the growth improvement caused by elevated sodium chloride is not due to a general osmotic effect.

In order to further compare the phenotypes of *mekk1* and *mpk4* single-mutant plants, we next tested the effects of elevated sodium chloride on the growth of *mpk4* mutants. As seen in figure 2A, the growth of *mpk4* plants declined with increasing levels of sodium chloride, and none of the sodium chloride concentrations tested improved the growth of *mpk4* plants. It was also observed that *mpk4* mutants are moderately resistant to 75 mM sodium chloride when compared to wild-type Landsberg (Fig. 2D). Although this difference was found to be statistically significant (t-test, $P<0.01$), it is not clear what the biological relevance of this mild salt resistance is.

In addition, we also tested the effect of sodium chloride treatment on the growth of mekk1 mutant plants that stably express a kinase-impaired version of MEKK1 under the transcriptional control of the *MEKK1* native promoter. We have previously shown that this kinase-impaired MEKK1 protein is able to rescue the mekk1 dwarf phenotype. (Suarez-Rodriguez et al., 2007) This mutant protein has a single amino acid substitution (K361M) in the ATP-binding pocket of the MEKK1 kinase. These K361M plants respond

to salt treatment in the same way that wild-type plants do, which provides further evidence that the kinase-impaired K361M protein is able to rescue the *mekk1* mutant phenotype.

mekk1 and mpk4 differentially affect lateral root formation

Another mutant phenotype that has been previously described for *mekk1* plants is a severe reduction in the density of lateral roots. (Nakagami et al., 2006) In order to determine if this phenotype is shared by *mpk4* plants we measured the number of lateral roots per centimeter in 11-day-old seedlings grown at 22^0C on vertically oriented agar plates for *mekk1-1*, *mpk4*, *mekk1-1* expressing the kinase-impaired MEKK1(K361M) protein, wild-type Columbia, and wild-type Landsberg (Fig. 3A). *mekk1-1* plants displayed an absence of detectable lateral roots, while *mpk4* plants produced an average of 2.9 lateral roots per centimeter (Fig. 3B). For comparison, wild-type Landsberg had an average of 3.6 lateral roots per centimeter. The *mpk4* mutation therefore causes a small, but significant (t-test, $P<0.01$), quantitative reduction in lateral root density relative to wild-type, whereas the *mekk1* mutation leads to the elimination of lateral root formation. *mekk1-1* plants expressing the MEKK1(K361M) protein had lateral root densities similar to that of wild-type Columbia, providing further evidence that the

kinase-impaired protein is able to rescue the *mekk1* mutant phenotype. The absence of lateral roots in *mekk1-1* plants suggests that the MEKK1 protein may function in a pathway that is important for lateral root production. To further explore this possibility we constructed a plasmid carrying a YFP-MEKK1 translational fusion under the transcriptional control of the *MEKK1* native promoter. This construct was introduced into *MEKK1/mekk1* heterozygous plants, and transgenic lines were isolated. Genotype analysis of the segregating progeny of these lines demonstrated that the YFP-MEKK1 fusion protein was able to rescue the *mekk1* mutant phenotype, indicating that the presence of the YFP tag did not interfere with the function of the MEKK1 protein (Fig. 3E,F). We next used epifluorescence microscopy to monitor the pattern of MEKK1 expression in these lines. Strong YFP-MEKK1 expression was observed in lateral root primordia, which is consistent with the idea that MEKK1 has a functional role in the production of these organs (Fig. 3C). The other region of the root that displayed strong YFP-MEKK1 expression was the root tip (Fig. 3C). A similar analysis of protein expression in root tissue was also performed for MPK4 using a GFP-MPK4 translational fusion expressed via the MPK4 native promoter. The expression pattern that we observed for MPK4 was similar to that of MEKK1, with the highest expression detected in lateral root tips and the primary root tip (Fig. 3D).

The mekk1;mpk4 double-mutant combination is seedling lethal

Previous work has indicated that MPK4 acts downstream of MEKK1. If these two proteins function exclusively in a linear signaling pathway then one would expect *mekk1;mpk4* double-mutants to be phenotypically identical to *mpk4* single-mutants. To test this hypothesis, we constructed plants that were double-mutant for *mekk1-1* and *mpk4*. Because *MEKK1* and *MPK4* are genetically linked, we first isolated plants with the genotype *mekk1-1/mekk1-1;MPK4/mpk4* in order to improve the efficiency with which we could generate homozygous double-mutant progeny. The *mekk1-1/mekk1-1*; *MPK4/mpk4* plants are not fertile in regular growth temperature (22-24^{0}C). Therefore, the plants are growing under the 28^{0}C. These plants were allowed to self-pollinate, and segregation at the *MPK4* locus was analyzed by PCR-based genotyping. One hundred and thirty seven seeds were plated onto agar plates, and 108 germinated seedlings were genotyped after seven days of growth at 22^{0}C. Fifteen percent of the seedlings tested were homozygous double-mutant, which is significantly less than the 25% that one would predict due to standard Mendelian segregation (Fig. 4). All 16 of the *mekk1-1/mekk1-1;mpk4/mpk4* seedlings that we identified displayed a seedling-lethal phenotype that was characterized by the absence of detectable root growth and an absence of true leaf development (Fig. 4C). If *mekk1-1/mekk1-1;mpk4/mpk4* seedlings

are left on the agar plate for longer periods of time no growth occurs, and the seedlings eventually die. Since ca. 18% of the seeds that were plated failed to germinate, it seems likely that the reduced number of homozygous double-mutant seedlings that we observed could be at least partially due to the failure of many of the homozygous double-mutants to germinate. No differences in growth were observed between plants with the *mekk1-1/mekk1-1;MPK4/mpk4* and *mekk1-1/mekk1-1; MPK4/MPK4* genotypes. Since we have shown that the dwarf phenotype of both *mekk1* and *mpk4* single-mutant plants can be largely rescued by growth at 30^0C, we next tested the ability of elevated temperature to rescue the seedling lethal phenotype of homozygous double-mutants. For this experiment seed was collected from an *mekk1-1/mekk1-1;MPK4/mpk4* parent plant, sown on soil, and incubated at 22^0C for five days under constant light to allow germination. The plants were then transferred to 30^0C for an additional three weeks of growth. Although growth of the homozygous double-mutant plants was improved by elevated temperature, when compared to their *mekk1* single-mutant sibs the double-mutants were much smaller in size (Fig. 4D–E). In addition, the homozygous double-mutants were not able to produce an inflorescence.

For comparison, the *mekk1* single-mutant plants produce a flowering bolt that is able to set seed when incubated at 28^0C. These results indicated that the *mekk1;mpk4*

double-mutant phenotype is much more severe than that of either single-mutant phenotypes.

DISCUSSION

Previous work describing *mekk1* and *mpk4* single-mutant plants has highlighted the similarity of the phenotypes caused by these mutations. This phenotypic similarity is consistent with the extensive biochemical analyses that have placed *MEKK1* upstream of *MPK4* in a MAP kinase cascade that is induced by the treatment of seedlings with the flagellin elicitor peptide flg22. (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007) Here we have documented an additional phenotypic similarity between *mpk4* and *mekk1* plants by showing that the dwarfism of *mpk4* plants can be rescued by high-temperature growth in the same way that the *mekk1* dwarf phenotype is rescued. This finding suggests that high temperature rescue occurs via a mechanism that involves a process downstream of *MPK4*. This result also lends further support to the idea that *MEKK1* and *MPK4* function in a shared signaling pathway.

We also observed, however, that there are significant differences in the phenotypes displayed by *mekk1* and *mpk4* single-mutant plants. First, we demonstrated that *mekk1* plants are partially rescued by growth on elevated levels of sodium chloride, while

mpk4 plants are not. The mechanism by which elevated sodium chloride is able to improve the growth of *mekk1* plants is not clear. One possibility is that salt-stress induces molecular changes in the *mekk1* plants that somehow correct for the imbalances created by the *mekk1* lesion, resulting in a net improvement in overall plant growth. Since elevated sodium chloride levels improve the growth of *mekk1* mutants, one could also postulate that *MEKK1* functions as a negative regulator of salt stress response. This model would seem to be at odds with the observation that *MEKK1* transcription is induced by sodium chloride treatment. (Mizoguchi et al., 1996) Due to the pleiotropic effects of the *mekk1* mutation, it seems more likely that the growth improvement observed in response to salt treatment may not be directly tied to signaling pathways that are specifically controlled by *MEKK1*. Another contrast in mutant phenotypes between *mekk1* and *mpk4* was observed in terms of lateral root formation. We observed that the *mpk4* mutation causes only a slight reduction in lateral root density when compared to wild-type, which contrasts sharply with the severe reduction in lateral root formation in *mekk1* plants. (Nakagami et al., 2006) Analysis of tissue-specific protein expression indicated that *MPK4* and *MEKK1* have very similar expression patterns, with both proteins most highly expressed in lateral root tips and in the primary root tip. These results suggest that *MEKK1* may play a unique role in lateral root formation, whereas the

function of *MPK4* in this process may be shared by additional MAP kinase isoforms that are able to compensate for the *mpk4* mutation via functional redundancy. The phenotypic differences between *mekk1* and *mpk4* plants suggest that the functionality of these two proteins is not limited to a single shared signaling pathway, because if this were the case then one would expect the two mutations to result in nearly identical phenotypes. The idea that MEKK1 and MPK4 function in more than one signaling pathway is further supported by the seedling-lethal phenotype displayed by double-mutant plants. At standard Arabidopsis growth temperatures, we observed that homozygous double-mutants arrested growth immediately following germination. No root growth was detected and no true leaves were formed. For comparison, both *mpk4* and *mekk1* single-mutant plants are able to grow long primary roots and survive in soil for several weeks. Although the true leaves of these single-mutant plants are small, they are nonetheless able to develop several pairs of true leaves. The double-mutant phenotype is therefore much more severe than either of the single-mutants.

We have previously demonstrated that MEKK1(K361M), which is a kinase-impaired version of MEKK1, is able to rescue the dwarf phenotype of *mekk1* plants. (Suarez-Rodriguez et al., 2007) This result suggests that structural features of MEKK1, such as the potential ability to function as a scaffold protein, may be more critical to its

function than the protein kinase activity. In the present study we have further characterized the ability of kinase inactive MEKK1(K361M) to rescue the *mekk1* mutant phenotype and have shown that the kinase-impaired protein can restore normal salt sensitivity and lateral root formation to the *mekk1* mutant. These findings indicate that the kinase activity of MEKK1 is dispensable for most if not all aspects of MEKK1-regulated signaling pathways. This is not to say that the kinase activity of MEKK1 is not involved in signaling in a wild-type plant, but rather that Arabidopsis may have redundant mechanisms that compensate for the missing kinase activity in MEKK1(K361M) plants.

From a genetic standpoint, *mekk1* and *mpk4* can be described as displaying synthetic lethality. The phenomena of synthetic lethality can be used to infer that two genes function in parallel signaling pathways that affect a biological process that is essential for viability. (Ooi et al., 2006) A simple model could be proposed where MEKK1 and MPK4 function in a shared MAP kinase cascade that is activated in response to the treatment of plants with flagellin (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007), and in addition to this shared pathway, each protein also participates in additional pathways that converge to regulate a process that is essential for post germination growth of seedlings. Further work will be needed to fully understand

the identity of these putative additional pathways through which each of these proteins function. It is apparent, however, that signaling via MEKK1 and MPK4 must involve more than just a single, linear kinase cascade. Continued application of double-mutant analyses between the many loci encoding components of MAP kinase cascades in Arabidopsis should allow for the construction of a detailed genetic interaction map that should help improve our understanding of the complexity present in MAP kinase signaling in plants.

MATERIALS and METHODS

Plant material

mekk1-1 (At4g08500; SK_052557) in the Columbia background and *mpk4* in the Landsberg background were grown in soil under constant light at 22^0C or 30^0C. A transgenic line stably expressing the kinase-impaired MEKK1(K361M) via the *MEKK1* native promoter in the *mekk1-1* homozygous mutant background has been previously described. (Suarez-Rodriguez et al., 2007) All analyses of *mpk4* mutant plants were performed using seed collected from homozygous *mpk4* parent plants. For the experiments involving *mekk1-1* mutant plants, seed was collected from a heterozygous

parent and all progeny were genotyped using PCR to identify homozygous *mekk1-1* individuals. For lateral root analysis seedlings were grown on vertically oriented plates containing 0.5x Murashige and Skoog basal salt mixture media with 0.7% agar (w/v). Plates were incubated under constant light at 22^0C for 11 days.

Genotyping

The following primers were used for PCR-based genotyping: MEKK1F: 5'-ATATCTCTCTCTTCGCCGTCAGGTTTTG-3', MEKK1R: 5'-AACTAGTAGGCTCCG GAAACTCCAAAGAG-3', p745: 5'-AACGTCCGCAATGTGTTATTAGTTGTC-3', MPK4F: 5'-GGAATTGTCTGGTGAGTTCCA-3', MPK4R: 5'-CCAGAGATGCTGATGAT TAGT-3', DS2: 5'-TTTCGTCGGTA ATCACCATTC-3'. The p745 primer recognizes the left border of the T-DNA insertion in *mekk1-1*, and DS2 recognizes the Ds transposon insertion in *mpk4*.

Osmotic stress treatments

Surface-sterilized seeds were placed on plates containing 0.5x Murashige and Skoog basal salt mixture with 0.7% agar (w/v) and incubated in the dark for five days at 4^0C. Following stratification the plates were incubated under constant light at 22 ^{0}C for 11 days. For sodium chloride treatment the growth medium was supplemented with 0 mM,

25 mM, 50 mM, 75 mM, or 100 mM sodium chloride. Whole seedling fresh weight, not including root tissue, was then determined. The additional osmotic stress treatments tested were 1%, 2%, 5%, and 10% sucrose; 1%, 2%, 5%, and 10% glucose; and 25 mM, 50 mM, 75 mM, and 100 mM mannitol.

Plasmid construction

A fragment of genomic DNA containing the *MEKK1* locus was PCR amplified from the wild-type Columbia genome and cloned into a plasmid vector. The *MEKK1* promoter region present in this clone includes 1730 bases of sequence upstream of the *MEKK1* start codon. Site directed mutagenesis was then used to introduce unique restriction sites immediately downstream of the *MEKK1* start codon in this clone. The coding region of the yellow fluorescent protein YFP was then PCR amplified and cloned into these restriction sites. The resulting *YFP-MEKK1* clone constitutes a translational fusion in which the YFP domain is present at the n-terminus of MEKK1. Expression of this construct is driven by the *MEKK1* native promoter. The resulting construct was moved into the binary vector pCAMBIA1300 and introduced into Arabidopsis via Agrobacterium-mediated transformation (Clough et al., 1998). The GFP-MPK4 construct was kindly provided by Morten Petersen and is comprised of 1150 bases of the promoter

region of the *MPK4* locus driving expression of an N-terminal GFP-MPK4 fusion in the pCAMBIA3300 binary vector.

Microscopy analysis

The imaging of YFP-MEKK1 and GFP-MPK4 transgenic plants was performed using an OLYMPUS B·60 compound microscope equipped for epifluorescence analysis of YFP and GFP fluorescence.

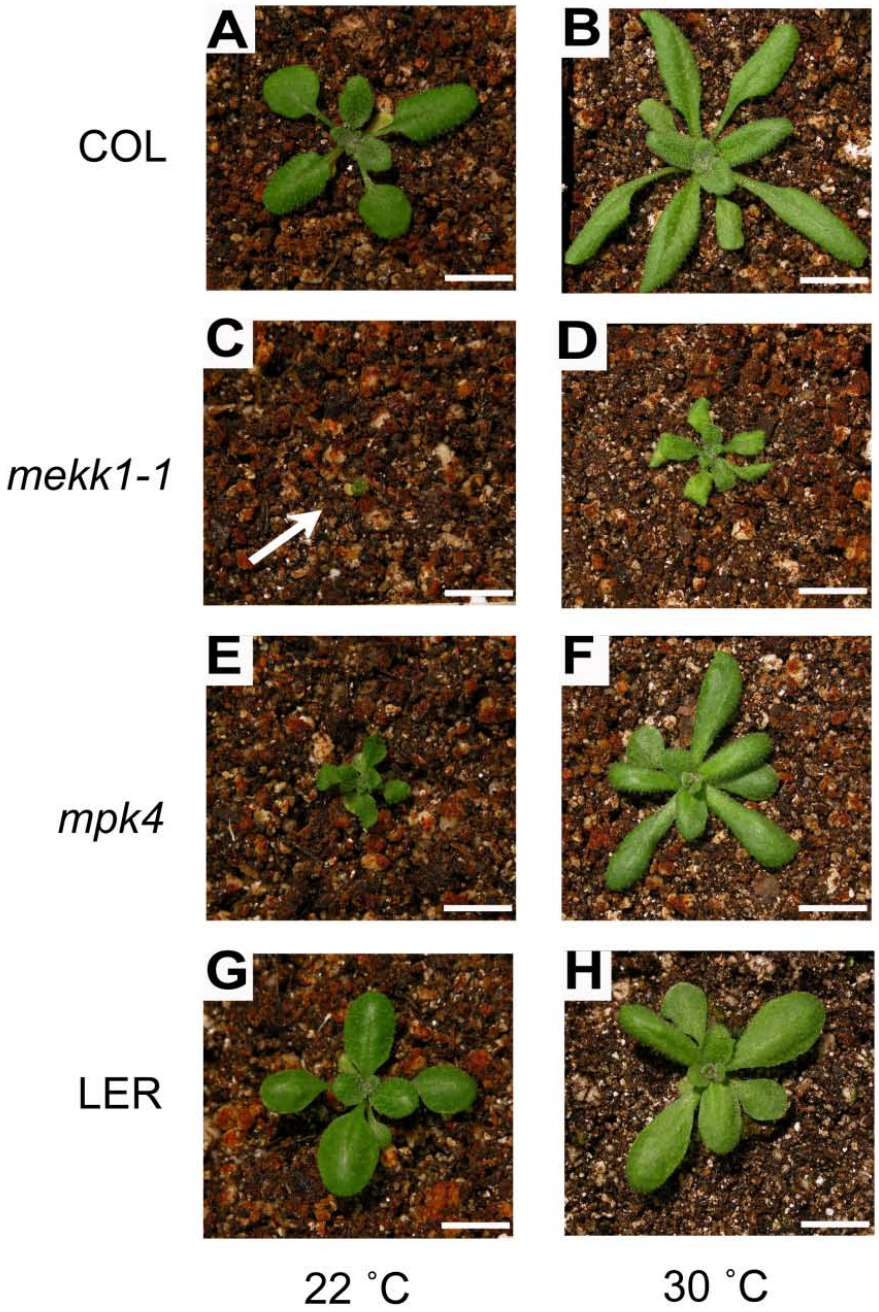

A
B
COL
C
D
mekk1-1
E
F
mpk4
G
H
LER
22 ˚C
30 ˚C

Fig. 1. *mekk1* and *mpk4* dwarf phenotypes are rescued by growth at high temperature. Two-week-old plants grown on soil at 22^0C (A, C, E, and G) or 30^0C (B, D, F, and H). (A, B) wild-type Columbia. (C, D) *mekk1-1*. (E, F) *mpk4*. (G, H) wild-type Landsberg erecta. Bars equal 1 cm.

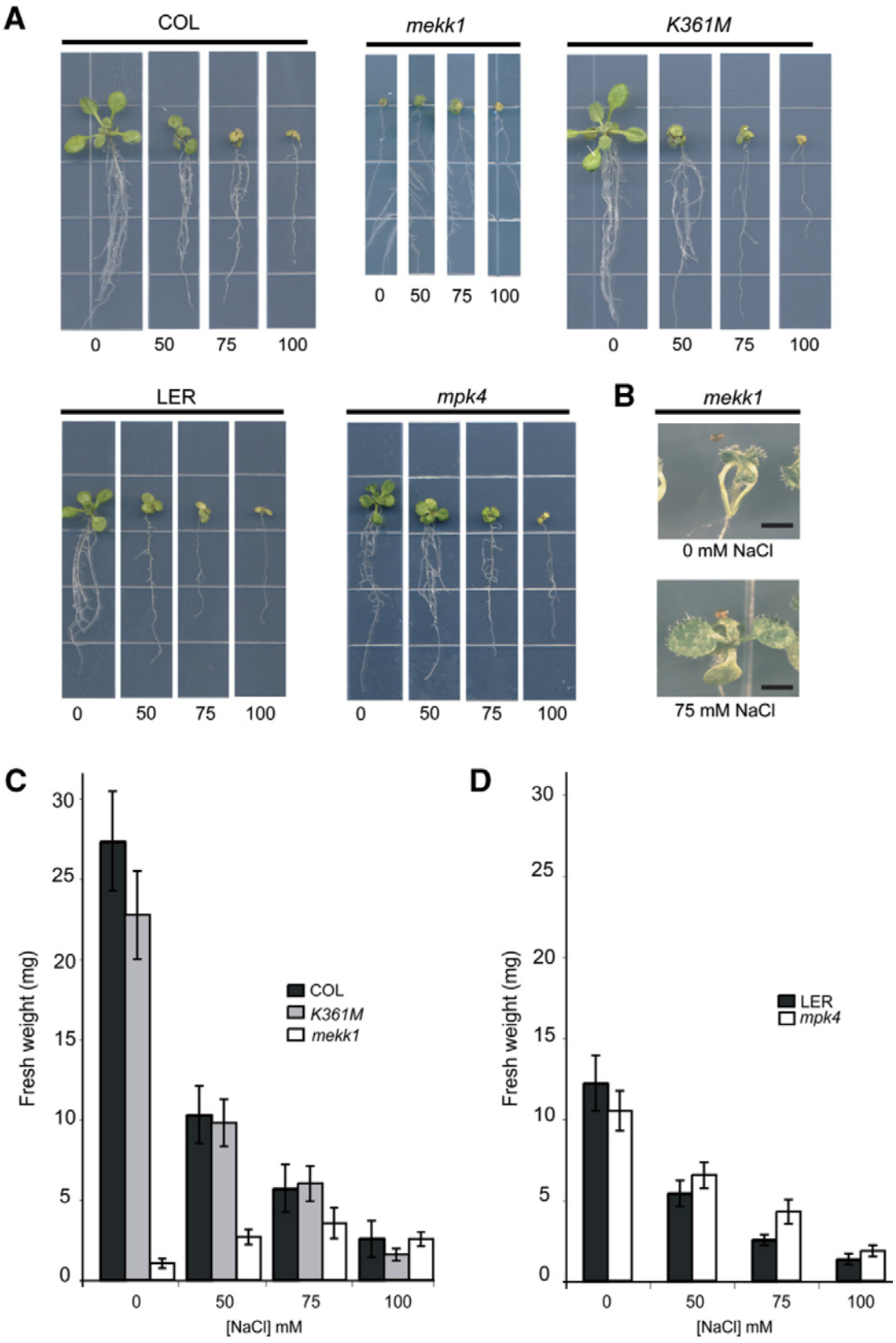

A
COL
mekk1
K361M
0 50 75 100
LER
mpk4
B
mekk1
0 mM NaCl
75 mM NaCl
C
Fresh weight (mg)
COL
K361M
mekk1
[NaCl] mM
D
LER
mpk4

Fig. 2. Sodium chloride treatment improves the growth of *mekk1* plants but not that of *mpk4* plants. Plants were grown for fourteen days on media supplemented with various concentration of sodium chloride at 22^0C under constant light. (A) Representative wild-type Columbia (COL), homozygous *mekk1-1 (mekk1)*, homozygous *mekk1-1* expressing the kinase-impaired MEKK1 (K361M) construct (K361M), wild-type Landsberg erecta (LER), and homozygous *mpk4* plants (*mpk4*). 0, 50, 75, and 100 indicate the concentration in millimoles of supplemental sodium chloride present in the growth media. (B) Close-up view of homozygous *mekk1-1* plants grown on 0 mM and 75 mM sodium chloride. Bars equal 2 mm. (C, D) Fresh weights of green tissue from two-week-old plants: (C) wild-type Columbia (n = 15–17), *mekk1* (n = 9–13), and K361M (n = 15–19), (D) wildtype Landsberg *erecta* (n = 14–19) and *mpk4* (n = 15–20).

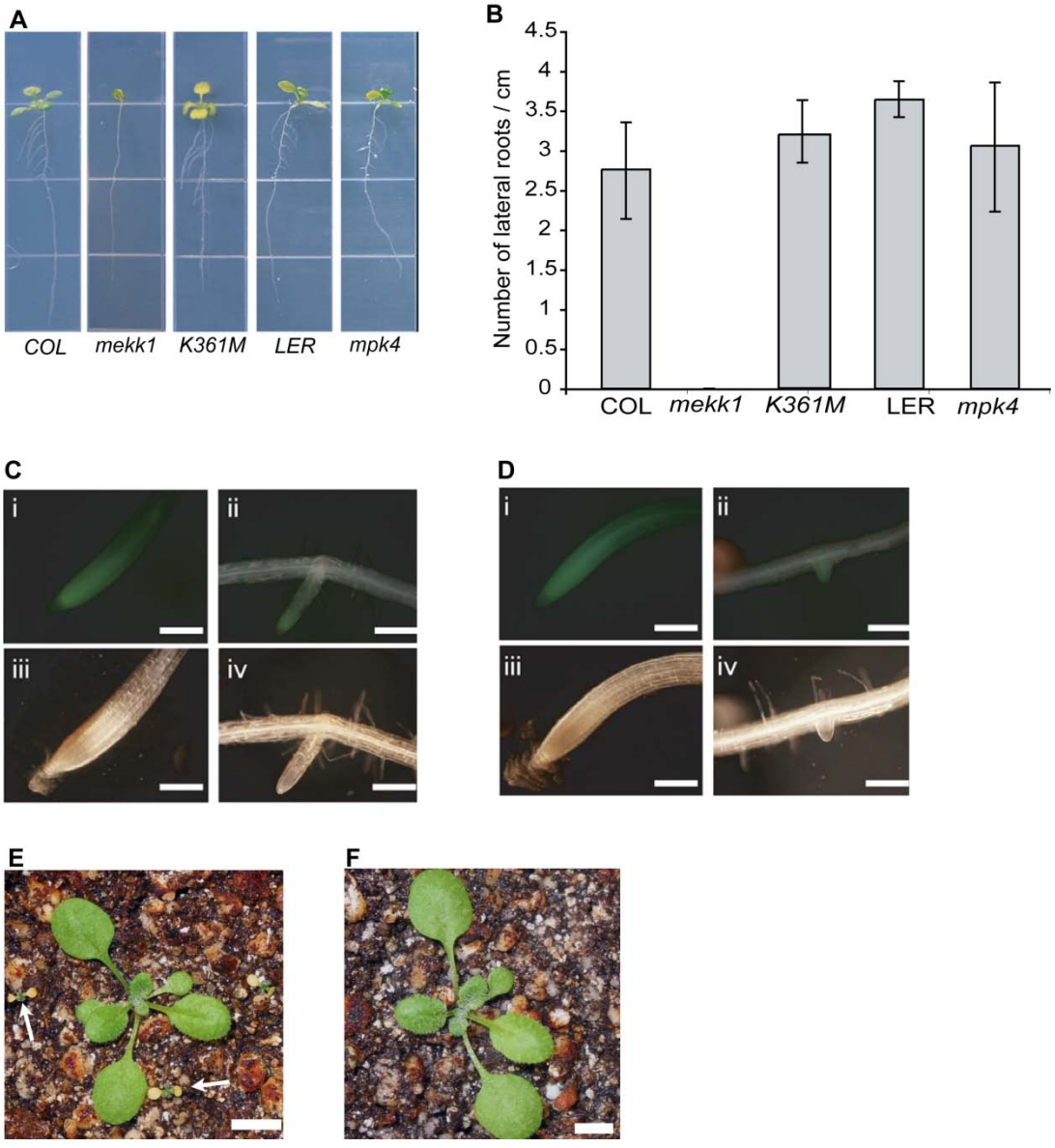
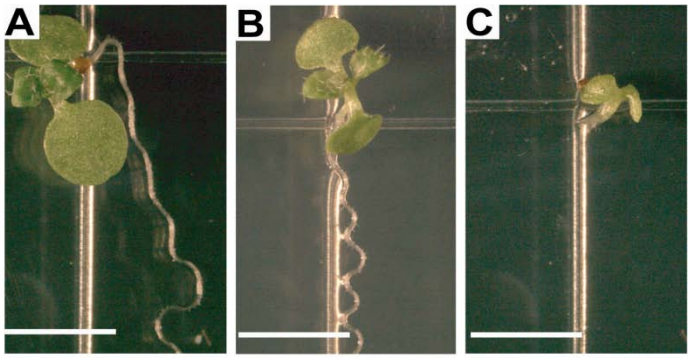
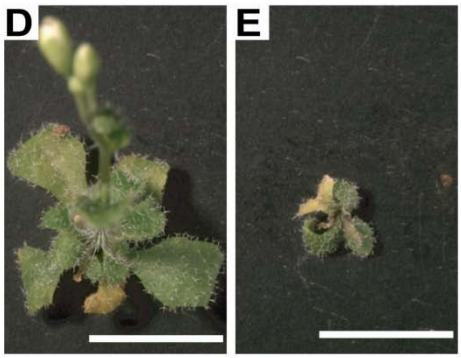

A
COL
mekk1
K361M
LER
mpk4
B
Number of lateral roots / cm
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
COL
mekk1
K361M
LER
mpk4
C
i
ii
iii
iv
D
i
ii
iii
iv
E
F

Fig. 3. Lateral root formation is inhibited in *mekk1* but not *mpk4* plants. (A) Representative 11-day-old plants grown on basal media under constant light at 22^0C. (B) Lateral root density. (n = 17–25). *K361M* indicates homozygous *mekk1-1* plants that express the kinase-impaired *MEKK1 (K361M)* construct. COL and *K361M* are not significantly different (t-test, P > 0.01). LER and *mpk4* are significantly different (t-test, *P*< 0.01). No lateral roots are produced by homozygous *mekk1-1* plants. (C,D) GFP- and YFP-fusion protein expression in root tissue driven by each gene's native promoter: (C) *MEKK1::YFP-MEKK1* and (D) *MPK4::GFP-MPK4*. i and ii are epifluorescence images. iii and iv are brightfield images. i and iii are primary root tips. ii and iv show lateral roots. Scale bars equal 0.5 mm. (E,F) Two-week-old plants grown on the soil at 22^0C under constant light. Bars are 0.5 cm: (E) wild-type Columbia and homozygous *mekk1-1* (white arrow). (F) Homozygous *mekk1-1* mutant expressing the *MEKK1::YFP-MEKK1* construct.



	MPK4/MPK4 mekk1/mekk1	*mpk4/MPK4 mekk1/mekk1*	*mpk4/mpk4 mekk1/mekk1*
Segregation ratio:	*37 (34%)*	*55 (51%)*	*16 (15%)*



MPK4/MPK4 mekk1/mekk1 30 °C

mpk4/mpk4 mekk1/mekk1 30 °C

Fig. 4. The *mekk1;mpk4* double-mutant combination causes seedling lethality. Seeds collected from an *mekk1/mekk1;mpk4/MPK4* plant were grown for seven days on agar plates at 22^0C and photographed. The genotypes of 108 individual seedlings were then determined by PCR. A representative plant for each genotype is shown. (A)*mekk1/mekk1;MPK4/MPK4*. (B)*mekk1/mekk1;mpk4/MPK4*. (C)*mekk1/mekk1; mpk4/ mpk4.* Bars are 0.5 cm. (D–E) Plants grown on soil at 30^0C for 26 days. A representative individual for each genotype is shown. Bars are 1 cm.

LITERATURE CITED

Jonak, C., Okresz, L., Bogre, L. and Hirt, H. (2002) Complexity, cross talk and integration of plant MAP kinase signalling. Curr. Opin. Plant Biol. 5, 415–424.

Mishra, N.S., Tuteja, R. and Tuteja, N. (2006) Signaling through MAP kinase networks in plants. Arch. Biochem. Biophys. 452, 55–68.

Tena, G., Asai, T., Chiu, W.L. and Sheen, J. (2001) Plant mitogen-activated protein kinase signaling cascades. Curr. Opin. Plant Biol. 4, 392–400.

Ichimura, K., Casais, C., Peck, S.C., Shinozaki, K. and Shirasu, K. (2006) MEKK1 is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. J. Biol. Chem. 281, 36969–36976.

Nakagami, H., Soukupova, H., Schikora, A., Zarsky, V. and Hirt, H. (2006) A Mitogen-activated protein kinase kinase kinase mediates reactive oxygen species homeostasis in Arabidopsis. J. Biol. Chem. 281, 38697–38704.

Suarez-Rodriguez, M.C. et al. (2007) MEKK1 is required for flg22-induced MPK4 activation in Arabidopsis plants. Plant Physiol. 143, 661–669.

Brodersen, P. et al. (2006) Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. Plant J. 47, 532–546.

Petersen, M. et al. (2000) Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell 103, 1111–1120.

Meszaros, T. et al. (2006) The Arabidopsis MAP kinase kinase MKK1 participates in defence responses to the bacterial elicitor flagellin. Plant J. 48, 485–498.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T. and Felix, G. (2006) The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell 18, 465–476.

Droillard, M.J., Boudsocq, M., Barbier-Brygoo, H. and Lauriere, C. (2004) Involvement of MPK4 in osmotic stress response pathways in cell suspensions and plantlets of Arabidopsis thaliana: activation by hypoosmolarity and negative role in hyperosmolarity tolerance. FEBS Lett. 574, 42–48.

MAPK Group (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci. 7, 301–308.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi- Shinozaki, K., Matsumoto, K. and Shinozaki, K. (1996) A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA 93, 765–769.

Ooi, S.L. et al. (2006) Global synthetic-lethality analysis and yeast functional profiling. Trends Genet. 22, 56–63.

Chapter 3

Developing a Methodology for Performing High-throughput Systematic Genetic Interaction Analysis in Arabidopsis

ABSTRACT

The overall goal of my research is to use genetic interaction analysis to study MAPK signaling pathways in Arabidopsis. In order to accomplish this goal, I decided to use a systematic approach that would involve generating large numbers of double-mutant Arabidopsis plants to search for evidence of genetic interaction. In order to complete this project in a reasonable time-frame, it was necessary to first concentrate on developing efficient methods for producing large numbers of homozygous double-mutant Arabidopsis plants for phenotypic testing. In this chapter I describe the resulting double-mutant generating pipeline that I developed. There are two high-throughput methods that form critical parts of this pipeline: (1) a system developed in our lab for performing high-throughput seedling growth and tissue collection, and (2) a method for performing gel-free PCR-based genotyping. Using this pipeline, I determined that one scientist can efficiently generate ca. 32 double-homozygous mutant lines every 2 weeks. For my genetic interaction studies, I built a target library composed of 75 single-mutant lines that target genes related to MAPK pathways including MAP3Ks, MAP2Ks, MAPKs and phosphatases. To query this target library for genetic interactions, I chose mutants in the three best-studied Arabidopsis MAPKs: *MPK3*, *MPK4*, and *MPK6*. I systematically created double-mutants between each of these query mutants and all 75

members of the target library. Furthermore, I also applied our pipeline to produce all possible double-mutants within a group of 13 MAPK genes. In total, I produced 287 double-mutants for further phenotypic assays. To identify evidence of genetic interaction, I performed a quantitative root growth rate assay as well as a visual screen for developmental phenotypes. This chapter focuses on the methodology of the high-throughput double-mutant pipeline that I developed. Details of the genetic interaction results that I obtained from this pipeline are described in chapter 4.

INTRODUCTION

In chapter 2, I described a series of genetic and phenotypic analyses of the MAP3K gene *MEKK1* and the MAPK gene *MPK4.* This work demonstrated the complexity of the functional relationship between these two *MAPK*-related genes in Arabidopsis. As mentioned in chapter 1, there are ca. 120 genes that encode kinases related to MAPK cascades in Arabidopsis. The functions of and the relationships between most of these genes are still unclear. According to recent studies in yeast, mapping the comprehensive genetic interaction network of an organism can help resolve complex signaling pathways into conceptually and experimentally tractable modules (Tong et al., 2004; Lin et al., 2008). Therefore, studying the genetic interactions between large numbers of gene pairs may help us to understand more about the functions of single genes and also the functional relationships between genes.

In contrast to traditional single-gene reverse genetic studies, there are no published reports describing systematic genetic interaction analyses in Arabidopsis to date. However, there are several alternative strategies that have been published to systematically study MAPK-related genes in Arabidopsis. First, comprehensive yeast two-hybrid assays were performed to test protein-protein interactions between all 10 of the MAP2Ks and all 20 of the MAPKs in Arabidopsis (Lee et al., 2008). In that study, 24

interactions were identified from 200 possible combinations. In the same study, *in vitro* kinase assays were also performed to examine the enzyme-substrate relationship of the interactors. Not all of the protein-protein interaction pairs showed an enzyme-substrate relationship (Lee et al., 2008).

Another large-scale analysis that has been reported involved screening for substrates of activated MAP kinases using high-density protein microarrays (Popescu et al., 2008). In order to generate active forms of the MAP kinase proteins for substrate screening, a comprehensive *in vitro* kinase assay between MAP2Ks and MAPKs was performed. In this study, forty-nine MAP2K-MAPK pairs were identified in which the MAP2K was able to phosphorylate the MAPK *in vitro.* Using these activated MAP kinase proteins to query their protein microarray, the authors identified 570 proteins as substrates of these Arabidopsis MAP kinases, with transcription factors representing a large portion of these proteins. These transcription factors were associated in large part with development and stress response (Popescu et al., 2008). These results provide support for prior evidence that MAPK signaling pathways regulate different biological processes related to development and stress response. This data also revealed that most these substrates are specific to one MAPK, and only small set of substrates can be phosphorylated by two or more MAPKs (Popescu et al., 2008).

These two comprehensive analyses have provided information regarding the biochemical properties of the proteins involved in MAPK signaling pathways in Arabidopsis. However, the kinase activity assays in these two studies were performed *in vitro*; therefore, it remains to be determined how well this information relates to the signaling that occurs in an intact plant.

More recently, a comprehensive transcriptional analysis of MAPK-related genes was performed in Arabidopsis (Menges et al., 2008). In that study, the authors analyzed the gene expression data for 114 MAPK-related genes from a microarray database and clustered the genes based on the correlation of their expression level during different development stages, in different organs, and under different treatments. These co-expression patterns were studied and mapped to make a gene expression atlas for MAPK-related genes. Since genes involved in the same biological process may show similar expression patterns in general or under treatment, the resulting information may provide the opportunity to predict possible functional roles for some of these MAPK pathway genes.

For my studies I was interested in using a genetic approach to systematically investigate gene function within the MAPK pathways in Arabidopsis. In order to accomplish this goal, I developed a new methodology for performing large-scale genetic

interaction analysis in Arabidopsis. This methodology includes optimization of the processes of double-mutant production and phenotypic assessment. By applying this new method, I was able to perform a pilot study investigating genetic interaction within genes related to MAPK pathways. This chapter describes the technical details of the novel methodology that I developed, while the following chapter describes the genetic interaction data I produced using this pipeline.

RESULTS

Overview of the genetic interaction pipeline

Based on previous work in other model organisms, three major steps are involved in systematic genetic interaction analysis: (1) **choose genes for screening**, (2) **generate double-mutant lines**, and (3) **phenotype the double-mutants to identify genetic interactions**. The general strategy of my pipeline is shown in figure 1.

Choosing genes for the study

Large-scale genetic interaction studies involve the use of two different groups of genes: **query genes** and **target genes**. A **query gene** is the gene for which the scientist wants to find interactors. The **target genes** are a large group of genes that

serve as library against which query genes will be screened. The corresponding single-mutants of query genes and target genes are called query mutants and target mutants. To easily understand large-scale genetic interaction analysis, we can compare it to the process of fishing: query genes are the bait and the fish swimming around in lake are target genes. Using different baits, as well as increasing the density of fish in the lake, could allow the angler to capture more fish. Ideally, the best scenario would be to use all of the genes from the genome as query genes and have all the genes from the genome in the target library. However, with current technology and resources, it is impractical to include all of the genes from the Arabidopsis genome in this type of analysis. Therefore, determining the subset of genes to be used as queries and targets is a very important step in the screening process.

Genes used in my large-scale genetic interaction analysis of MAPK cascades in Arabidopsis

Query Genes. For my large-scale genetic interaction analysis, I focused on searching for interactors of three well-studied MAPK genes: *MPK3*, *MPK4*, and *MPK6*. These three genes are the query genes in my project. *MPK3* and *MPK6* are closely related homologues, and there are no obvious abnormal phenotypes identified in *mpk3* or *mpk6*

single-mutants in the vegetative growth stage (Wang et al., 2007). However, *mpk6* plants show reduced fertility due to defects in anther and ovule development (Bush and Krysan, 2007; Wang et al., 2007). By contrast, *mpk4* plants show an abnormal phenotype at the seedling stage: roots are shorter than wild-type, the shoot area is smaller, and the cotyledons turn yellow and bend downward. Mature *mpk4* plants show severe dwarfism and infertility (Peterson et al., 2000; Kosetsu et al., 2010).

Target library. Based on the Arabidopsis genome annotation from the TAIR genome database version 10, there are 27,416 predicted genes in the Arabidopsis genome (The Arabiodpsis Information Resource, 2011). Currently, 65% of these genes have homozygous T-DNA insertion alleles available (http://signal.salk.edu/gabout.html; Alonso et al., 2003). However, because of the time required for producing double-mutants in Arabidopsis, it is impractical to use all available T-DNA mutant lines in the target library for this analysis. For this reason, properly defining a subset of genes for the target library is necessary for this study.

In my project, I am interested in understanding MAPK signaling pathways. As mentioned in chapter 1, the typical signal transduction within these pathways is based on sequential phosphorylation between three categories of kinases, MAP3K, MAP2K, and MPK. Dephosphorylation by phosphatases is also an important regulatory process that

affects MAPK pathways (Widmann et al., 1998). Based on results of a previous study in yeast, genes belonging to the same pathway or parallel pathways tend to be more likely to show genetic interactions with each other (Tong et al., 2004). Therefore, by focusing our target library on a subset of protein kinases could potentially increase the odds of identifying genetic interactions in my study.

To build a target library, I systematically obtained mutants in genes related to MAPK pathways from the homozygous T-DNA mutant collection from the SALK Institute (Alonso et al., 2003). I tested the T-DNA insertional mutant lines that I obtained from the homozygous collection using PCR-based genotyping to identify homozygous single-mutant individuals. In the target library used for this project, there are a total of 75 single-mutants representing four different groups of *MAPK*-related genes: 7 MAP4Ks, 47 MAP3Ks, 10 MAP2Ks, and 11 phosphatases, as presented in Table 1. Detailed information about each of these mutant lines is listed in Table 2.

MPK gene family. In the previous section, I described the genes that I used to perform query-target screening. For my project I also used a second strategy for producing double-mutants. The underlying rationale for this strategy is identical to that described in the query-target approach: generate double-mutants and phenotype them to search for the evidence of genetic interaction. The only difference for this second strategy is

the organizational strategy for planning which double-mutants to produce. Instead of using query mutants to cross with a target library, for this strategy each single-mutant within the MAPK gene family was crossed with all the MAPK single-mutants to produce all possible combinations of double-mutants within this group. There are 20 genes in the MPK gene family, but suitable T-DNA lines were only available for 13 of these genes based on the following criteria: (1) no full length mRNA was detected, and (2) no chromosomal translocation was present (Clark, 2010). The 13 T-DNA lines that pass both requirements are listed in Table 2. By crossing all of these 13 T-DNA lines with each other, I produced 74 double-mutants for further phenotypic study. A detailed analysis of these double-mutants will be described in the next chapter.

Generating double-mutants

Unlike previous studies in yeast in which cells could mate to produce double-mutants spontaneously, Arabidopsis plants require manual manipulation to produce double-mutants. The value of a genetic interaction screen is dependent on the scientists' ability to generate a large number of double-mutants; therefore, efficiently generating double-mutants for analysis is important. In general, production of a double-homozygous mutant starts with planting two single-mutant plants and

cross-pollinating those plants manually to produce the double-heterozygous mutant F1 plants. The F1 plants are allowed to self pollinate and produce F2 seeds. F2 seedlings which segregate at two loci are grown and genotyped to identify double-homozygous mutant plants. Then, double-homozygous mutant seeds (F3) are collected. Last of all, these F3 seeds are planted and the seedlings are analyzed to identify any phenotype suggesting a genetic interaction. (Fig. 1)

High-throughput methods that accelerate the double-mutant generating pipeline

As mentioned above, in order to obtain double-homozygous mutants, individual plants from a segregating F2 population must be genotyped using PCR. Based on Mendelian segregation, the rate of identifying double-homozygous mutants for two unlinked mutant alleles in a segregating population is 1 out of 16. In practice, the chance of getting a double-homozygous mutant in a population of F2 seedlings may be lower than this ratio. Therefore, the most effective strategy for efficiently identifying homozygous double-mutant plants is one that involves genotyping more than 16 seedlings from each F2 population. In my experience, the process of genotyping F2 seedlings to find homozygous double-mutant individuals is a bottleneck that limits productivity of genetic interaction analysis in Arabidopsis. To accelerate the process of

producing homozygous double-mutant plants, I incorporated two high-throughput methods into my pipeline: (1) high-throughput seedling growth, tissue collection, and DNA isolation using the Ice-Cap system, and (2) gel-free PCR-based genotyping.

*The Ice-Cap method***.** Ice-Cap is a method used for efficiently planting, growing, and harvesting seedlings 96-at-a-time (Krysan et al., 2004; Clark & Krysan 2007). The Ice-Cap system is based on the use of two 96-well plates for growing Arabidopsis seedlings, arranged as an upper plate and a lower plate. The upper plate is a 96-well spin column plate filled with agar media in which individual seeds are manually plated into each well. The lower plate is a 96-well PCR plate that is filled with water for root growth; each well of the lower plate also contains a steel ball. When root tissue penetrates through the agar in the upper block, it grows down into the lower plate. Then, root tissue is harvested by freezing the lower water plate. Once the water in the lower plate has frozen, the upper plate is peeled away from the lower plate and the root tissue is processed to obtain genomic DNA that can be used as a template for PCR-based genotyping. The seedlings in the upper plate remain viable through this process and can be stored at 4^0C for at least 4 weeks after root tissue has been harvested with no loss of seedling viability (Su et al., 2011). Based on my personal

experience, one scientist can harvest root tissue from 40 Ice-cap plates in an 8-hour day, which results in the purification of DNA from 3,840 individual seedlings in a single day.

Gel-free PCR-based genotyping. The second step that has the greatest potential to slow down the rate of production of homozygous double-mutant plants is the analysis of the large number of PCR reactions that are used to determine the genotype of each F2 seedling. In standard PCR-based genotyping, DNA samples from each seedling are analyzed using two separate PCR reactions for each loci that is segregating. One PCR reaction targets the T-DNA mutant allele of the gene, while a second reaction targets the wild-type allele. Consequently, to determine the genotype of a single seedling that comes from a population that is segregating at two different locus, four different PCR reactions are required. To reduce the labor and accelerate the screening process, I used a two-step genotyping strategy for my pipeline. Each DNA sample from the F2 population was initially tested using only two PCR reactions, rather than four. One reaction tested for the presence of the wild-type allele of the query gene, and the second tested for the presence of the wild-type allele of the target gene. I analyzed the results to identify candidate double-homozygous mutant plants that were lacking both amplicons. Seedlings that passed this initial screening step were considered potential double-homozygous mutants. However, technical failure of the PCR reaction could

also explain the lack of PCR products for these two reactions. Therefore, candidate double-homozygous mutant plants were retested to validate their genotypes using freshly prepared DNA extracted from candidate seedlings that had been transferred from the Ice-Cap plate to soil. In this case, four PCR reactions were performed on each candidate seedling to test for the mutant and wild-type alleles of both the query and target loci. In a typical screen, ca. eight candidate double-homozygous mutants were identified from the initial screening of the seedlings in one Ice-Cap plate. Follow-up genotyping typically resulted in the identification of four authentic double-homozygous mutants.

Typically, the analysis of PCR reactions for the presence or absence of a PCR amplicon requires electrophoresis of the reaction products through an agarose gel containing a DNA-binding dye such as ethidium bromide. Figure 2B shows the results of a representative PCR-based genotyping reaction on plants carrying a T-DNA mutant allele of the *MEKK3* locus using the electrophoresis method. Although it is an effective method for analyzing PCR reactions, using agarose gel electrophoresis to process the thousands of PCR reactions needed for my project would constitute a major bottleneck in my double-mutant pipeline. To avoid this bottleneck, I used melt curve analysis of PCR amplicons as an alternative to agarose gel electrophoresis. For melt-curve analysis, a

fluorescent dye called EvaGreen is included in each PCR reaction. EvaGreen can bind to double stranded DNA such as the PCR amplicon and the resulting fluorescence can be detected using the CFX96 PCR Detection System from Bio-Rad Laboratories. Different PCR amplicons show different melting temperatures or a unique shape of the melting curve due to differences in sequence composition and product size. These features allow us to determine the presence of a specific PCR product in a reaction without having to load individual PCR samples into an agarose gel. Melt-curve analysis can be performed in the same PCR plate that was used for performing the thermal cycling, with no additional manipulations required. One simply needs to move the PCR plate from the thermocycler machine to the melt-curve machine and run the melt-curve program. Figures 2C-E display representative results obtained when plants carrying a T-DNA mutant allele of the *MEKK3* gene were analyzed melt-curve analysis. Based on personal experience, one scientist can perform melt-curve analysis on 96 PCR reactions in 10 minutes and ca. 576 PCR reactions in an hour. Using this method to analyze PCR-based genotyping results, I can analyze the genotypes of 1,920 samples, or 20 F2 populations, in a single day.

Timeline of double-mutant production pipeline

I developed a standardized pipeline for producing homozygous double-mutant Arabidopsis plants, and the timeline is shown in figure 3. This pipeline is divided into three stages. **Stage one** involves producing double-heterozygous F1 mutant plants by manually cross-pollinating plants carrying a mutation in a target gene with pollen from a plant carrying the query mutation. This stage of the pipeline takes ca. 7 weeks from plating the parental single-mutants until the seeds derived from cross are collected. **Stage two** of the pipeline is the collection of F2 seeds from F1 double-heterozygous plants. This stage includes planting the F1 plants, genotyping the seedlings to confirm that they are heterozygous for both the query and target mutations, and collecting the resulting F2 seed from individual F1 plants. The genotype of F1 plants is confirmed by PCR-based genotyping. This stage of the pipeline takes 8 weeks to complete. Finally, **stage three** of the pipeline involves identifying double-homozygous mutants to analyze for genetic interaction. The major work in this stage of the pipeline is genotyping 96 individual progeny using the Ice-Cap system for each F2 population in order to identify double-homozygous mutants. Once a double-homozygous mutant has matured, the F3 seeds are collected. These F3 seeds are the material upon which quantitative and qualitative phenotyping will be performed in order to identify genetic interactions.

This pipeline, from the planting of two single-mutants through to the collection of F3 double-homozygous seeds, requires 6 months. However, during this 6 month time frame, only sporadic periods of labor are required. Plant growth occupies major portions of the six-month timeline. In my practical experience, a single researcher can easily and efficiently work with 32 double-mutant lines per cycle; and every two weeks a new cycle can be initiated. In other words, with the pipeline running at full capacity, there will be 32 double-mutants generated every two weeks. Based on this rate, ca. 768 double-mutants could be generated in one year.

Phenotyping the double-mutants to identify evidence of genetic interaction

As mentioned in chapter 1, genetic interaction can be described as the situation where the phenotype of a double-mutant cannot easily be explained by combining the phenotypes of the corresponding single-mutants. Therefore, after successfully generating a large number of double-mutants, selecting an appropriate method to characterize the phenotypes of these double-mutants is critical for identifying candidates of genetic interaction. Depending on the measurement of interest, the phenotyping system could be either quantitative or qualitative. Qualitative phenotypes are the features that cannot easily be represented numerically, such as leaf morphology or

flower morphology. Quantitative phenotypes, by contrast, are those which can be represented on a continuous numerical scale. For example, root length or hypocotyl length are quantitative phenotypes. Based on large-scale genetic interaction analyses that have been performed in other model organisms, there is evidence to show that quantitative phenotypic assays are more sensitive than qualitative assays for detecting differences between double-mutants and their corresponding single-mutants. Using a quantitative phenotyping strategy therefore has the potential to increase the chance of finding evidence for genetic interaction between a pair of genes (Byrn et al., 2007; Costanzo et al., 2010).

Phenotyping assay used in this project

For my project, I chose two basic phenotypic analyses: quantitative root growth measurement and qualitative developmental observation.

Quantitative root growth measurement. For the root growth analysis, each double-mutant was planted with its corresponding single-mutants and the wild-type Columbia-0 for comparison. To reduce the possibility of phenotypic difference due to variance in the growth environment of parental lines, all double-homozygous mutants and their corresponding single-mutants were grown in the same growth chamber under

the same growth conditions prior to collecting the seeds for quantitative phenotyping. For root growth measurement, seedlings were grown on vertically oriented agar plates. To quantify root growth, images of the seedlings were collected using a flatbed scanner four days and six days after being placed in the light. To eliminate variation caused by asynchronous germination, the rate of growth between day four and day six was determined and used as the phenotypic index, rather than end point analysis of root length. After collecting the data, the results were analyzed statistically using an unpaired t-test. The double-mutants showing a significant difference in growth rate, compared to both corresponding single-mutants, were considered to be candidates of genetic interaction. Further details of these results will be discussed in chapter 4.

Qualitative developmental observation. For this analysis, seedlings were transferred to soil and propagated in growth chamber conditions. The qualitative phenotypes of the plants that I surveyed included rosette leaf morphology, flower development, and fertility. In these observations, I was screening for obvious differences between the double-mutants and their corresponding single-mutants. The results from these observations are not intended to represent a comprehensive screen for all developmental differences, but rather constitute a broad screen for dramatic differences

in plant development. More details of these quantitative and qualitative phenotypic assays are discussed in chapter 4.

Special Case: Genetic interaction analysis in plants carrying a mutation in MPK4

Based on research in yeast, the density of the genetic interaction network of essential genes is five times greater than the density for non-essential genes (Davierwala et al., 2005). Consequently, I elected to use the *mpk4* mutant as a query mutant since plants homozygous for this mutation show a severe dwarf phenotype, suggesting that MPK4 is critical for normal plant development. Therefore, including MPK4 in my project may increase the chance of identifying genetic interactions. Because of the infertility of *mpk4* single-mutants, I could not collect progeny from double-homozygous mutants containing *mpk4* for phenotypic analysis. To overcome this situation, I used an alternative strategy for producing and analyzing double-mutants that contain mpk4. This strategy is described below.

Producing double-mutants with mpk4. Because Arabidopsis is a diploid organism, a scientist can maintain mutant alleles in the heterozygous state for homozygous mutants that are lethal or infertile. Thus, instead of identifying and collecting double-homozygous mutants from *mpk4* F2 populations, I focused on obtaining

seedlings which were homozygous mutant for each target gene and heterozygous for *mpk4.* The F3 seeds from these plants were collected for phenotypic analysis. In the resulting F3 population, the *mpk4* mutant allele is segregating, while the target mutation is homozygous. One would therefore expect 25% of the F3 seedlings to be double-homozygous mutants. The double-homozygous mutants that do not genetically interact with *mpk4* would be expected to show the same phenotype as an *mpk4* single homozygous mutant. Since *mpk4* single-mutants have a strong and easily distinguishable mutant phenotype that is detectable at the seedling stage, I can visually screen seedlings on the agar plates used to measure root growth and identify double-homozygous mutants in the F3 generation.

Phenotypic analysis. The general strategy for the phenotypic analysis of double-mutants containing *mpk4* is similar to that for other double-homozygous mutants. The only difference for this group is an additional step to identify the double-homozygous mutants from the segregating population on the root growth plates. The root growth plates for the populations containing *mpk4* are processed identically to those for standard homozygous double-mutants, as described above. Plates are scanned four and six days after transfer to light in order to measure root growth rate. Because seedlings that are homozygous for the *mpk4* mutation have a root that is significantly

shorter than wild-type, seedlings homozygous for *mpk4* can be easily identified on the root growth plates. This phenotype appears in 4-day-old seedlings and is very distinguishable on 6-day-old seedlings. Figure 4 shows typical 6-day-old seedlings from a population that is segregating the *mpk4* mutant allele.

For root growth analysis, segregating F3 seeds were plated on agar plates and the double-homozygous mutants were identified based on the root images captured on day 6. The quantitative data for root growth rates were only collected and calculated for the seedlings that displayed the *mpk4* root phenotype. Additionally, for the qualitative phenotypic assay, only double-homozygous seedlings with an abnormal root phenotype were transferred to soil. All of the following analyses for these *mpk4* mutants were the same as the regular phenotypic assays described in last section for double-mutants. It should be noted that if an alleviating genetic interaction was present in one of the double-mutant combinations, then one would expect to observe no seedlings that display the short root phenotype associated with the *mpk4* single-mutant condition. The reason for this is that all of the F3 populations used for analyzing genetic interactions with *MPK4* are homozygous for the target mutation. In other words, all of the progeny on the plate will be homozygous for the target mutation. If the target mutation rescues the mutant root phenotype of *mpk4*, then all of the seedlings would be rescued. For this reason,

visually screening for the *mpk4* short root phenotype in these F3 populations allows us to identify two different categories of genetic interactions: (1) those that make the roots grow more poorly than the *mpk4* single-mutant alone and (2) those that rescue the short root phenotype and make the roots grow better than the *mpk4* single-mutant alone.

Summary of double-mutant pipeline: 289 double-mutants were produced

In this project, I applied our high-throughput double-mutant producing pipeline and successfully generated 289 double-mutants using genes related to MAP kinase pathways. These double-mutants can be divided into two different categories: *Query-target pairs* and *MPK-MPK pairs*

Query-target pairs. In this category, I generated 213 double-mutants in total, from three query mutants and 75 target mutants. In each query mutant background, there are 71 double-mutants.

MPK-MPK pairs. I also generated double-mutants using a set of 13 single-mutant lines from the *MAPK* gene family. In this category, I produced 72 F2 populations in order to find homozygous double-mutants (Table 3). Two mutant combinations, *mpk1/mpk9* and *mpk3/mpk17*, were not identified in the F1 generation due to technical failure. In addition, two of the F2 populations generated for this project failed to yield

double-homozygous mutants. One is the *mpk6;mpk20* double-mutant, in which the two genes are closely linked on chromosome 2. My failure to find a homozygous double-mutant for this combination is likely due to the close linkage of these two loci, which makes the recombination event that is needed to bring the mutant alleles into the cis configuration a relatively rare event. It is likely that by screening more progeny from the F2 population one could identify the desired cis configuration off these two mutant loci, and then proceed to find the homozygous double-mutant. The other homozygous double-mutant that I failed to recover is *mpk3;mpk6*, which has been shown to be embryo lethal in a previous study (Wang et al., 2007).

The results of my large-scale genetic interaction screen are described in Chapter 4.

DISCUSSION

Studying large-scale genetic interaction has become an important experimental approach in different model organisms in the past several years. A major reason for this phenomenon is that systematic analysis of the genetic interaction network can provide useful information, not only to understand the functions of individual genes but also to understand the relationships between genes in different signaling pathways controlling a biological process (Tong et al., 2004; Schuldiner et al., 2005; Lin et al.,

2008). To perform systematic genetic interaction analyses, there are two important criteria. First, a large collection of defined single homozygous mutants or other reagents which can easily inactivate targeted gene (e.g. a collection of *E coli* strains for RNAi in *C. elegans*) is required. Second, an efficient method for systematically producing a large number of double-mutants for analysis is also necessary.

In Arabidopsis, there is a well-established T-DNA insertional mutant collection available through a database from the SALK Institute that provides easy access to single-mutant lines (Alonso et al., 2003; TAIR, 2011). However, when I began my project there was no method available for the efficient generation of large numbers of double-mutants. To overcome this problem, I developed a double-mutant producing pipeline that features two high-throughput methods that substantially increase the efficiency with which double-mutants can be generated: the Ice-Cap system for seedling growth and DNA extraction and a gel-free method for PCR genotyping. Using this novel pipeline, one scientist can not only systematically grow and organize a large number mutant plants but also efficiently genotype the seedlings to identify the double-mutants. This pipeline has the capacity to generate ca. 768 double-mutants per year per scientist. This rate makes studying large-scale genetic interaction practical in Arabidopsis. In the

following chapter, I will detail the results that I obtained from my phenotypic assays and discuss the gene pairs which show strong evidence of genetic interaction.

MATERIAL and METHODS

Plant material and growth condition

Arabidopsis thaliana plants were grown in soil under continuous light at 18-20^{0}C. Plant materials used in this work is all in the Columbia-0 (Col-0) ecotype. 88 T-DNA insertion single-mutants (Table 2) were used in this work and all the lines were ordered from ABRC stock center (http://abrc.osu.edu/; Alonso et al., 2003). Single-mutants were tested by PCR-based genotyping to confirm the T-DNA insertion location and genotype. All the primers used for genotyping are also listed in Table 2.

Ice-Cap seedling growth system

Seeds were grown using the Ice-Cap growth platform as described (Krysan 2004; Clark and Krysan 2007). Seeds were plated and grown on 96-well plates containing 0.6% agar (Sigma). Plants in Ice-Cap blocks were grown for 2 to 3 weeks in a continuous watering system under constant light. Roots grew down through the agar into a second 96-well plate filled with water. When roots reached the bottom, the bottom plates were

frozen in a dry ice-ethanol bath to capture the root tissue. Root samples were separated from the seedlings leaving viable plants on the upper plate. 25 microliters of a solution of 500 mM Tris, pH 8, 50 mM EDTA, pH 8, was added to the root plate. The plates were sealed, and root tissue was pulverized with steel beads by shaking for 3.5 min at 1500 strokes/minute. After centrifuging the plates at 3400 rpm for 10 minutes, the supernatant fluid was then diluted 1:5 in distilled water. The genomic DNA from this dilution was used as a PCR template for amplification and mutation scanning. After root extraction, the Ice-Cap plates containing the seedlings still growing in agar plates were sealed with tape and covered with foil for storage in the dark at 4°C for up to 4 weeks. Plants of interest were extracted from Ice-Cap wells and transplanted to soil with their associated agar plugs. These plants were grown under constant light at 20°C.

PCR-based genotyping and melting-curve analysis

Genomic DNA was used as a template for PCR. PCR reactions with a final volume of 20 uL contained 75 mM Tris-HCl, pH 9, 20 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.01% (v/v) Tween20, 0.2 mM of each deoxyribonucleotide triphosphate (Promega), 0.2 uM of each PCR primer, 2.5 uM EvaGreen nucleotide-binding dye, and a mutant form of Taq polymerase that has a reduced activity at room temperature (Kermekchiev et al. 2003). A

liquid-handling robot (SciClone ALH3000; Caliper LifeSciences) was used to dispense 2 uL of the DNA template into each PCR.

Primers used for PCR amplification are listed in Table 2. PCR products amplified for mutation detection were melted using the CFX96 PCR Detection System from Bio-Rad Laboratories. The SYBR/FAM emission/detection channel (450–530 nm) was used to detect fluorescence of EvaGreen bound to the PCR products.

Quantitative analysis

Double-mutants (F3) seeds were plated on 1% agar plates with 0.5x Murashige and Skoog media. After cold treatment for at least 48 hours to stratify the seeds, the plates were grown vertically under continuous light at 18-20°C. Root tip positions of 4-day-old seedlings were marked and subsequently images of 6-day-old seedlings were scanned at resolution of 600dpi by an Epson v700 scanner. The root length was measured using ImageJ software from NIH image group (Rasband, 1997-2011, http://imagej.nih.gov/ij/). The difference between 4-day and 6-day root length was calculated and used as the final index to determine the evidence of genetic interaction.

Outline of Double Mutant Generating Pipeline

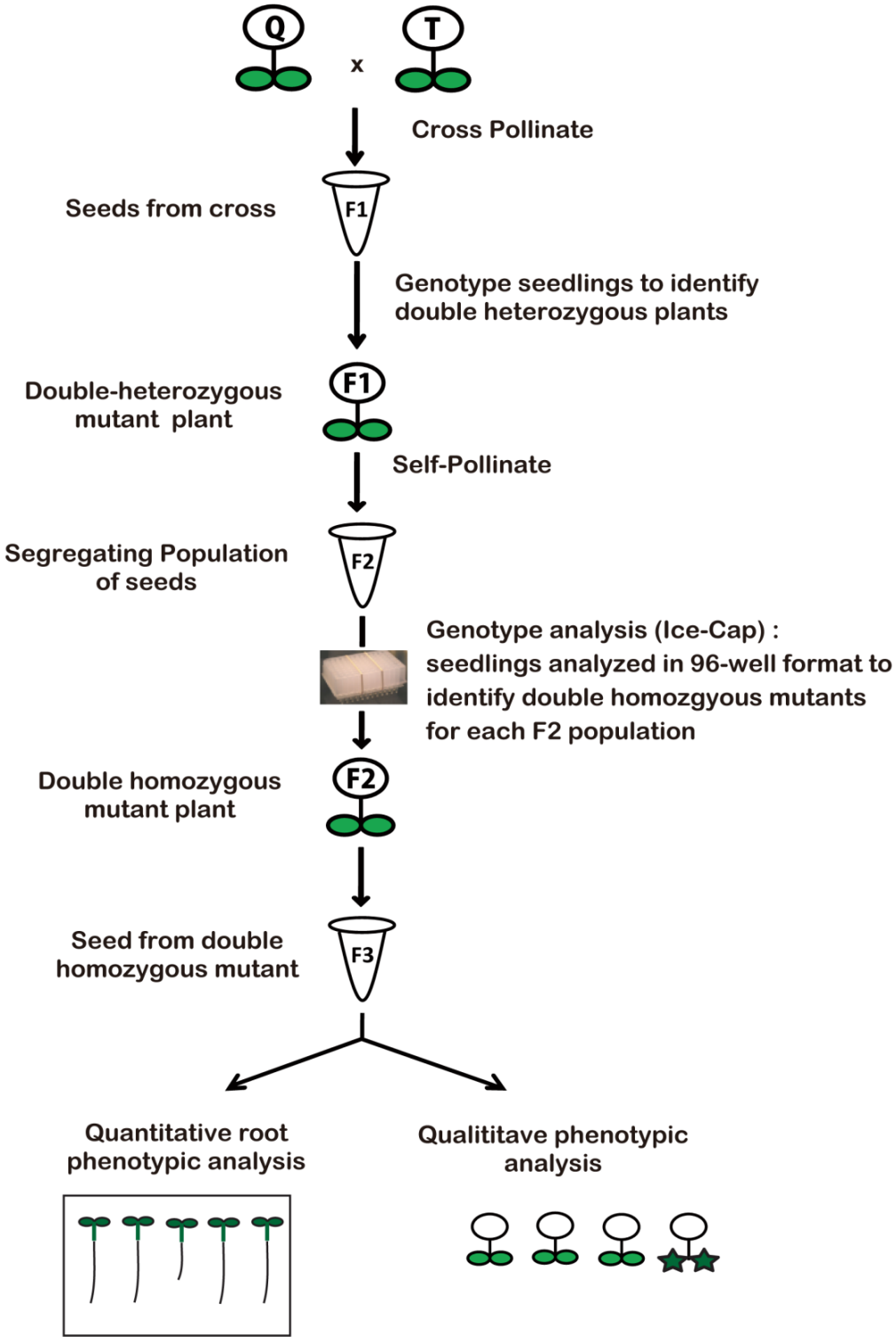


Fig. 1. Work flow of the double-mutant pipeline. Query (Q) and target (T) single-mutants are crossed to produce F1 double-heterozygous plants. The F1 plants are self-pollinated and produce F2 seeds in which the query and target mutations are segregating. F2 seeds are collected for genotyping by using Ice-Cap methods. F2 individuals that are homozygous double-mutant are transferred to soil and grown to maturity. Seed from the homozygous double-mutant plants (F3 seed) is collected. F3 double-mutant seedlings are screened for abnormal phenotypes both quantitatively and qualitatively.

Table 1. Gene families chosen for large-scale genetic interaction analysis.

Gene family	Biochemical function	Number of genes in gene family	Number of homozygous lines in this study
MAP4K	*Kinase*	*10*	*7*
MAP3K	*Kinase*	*80*	*34*
MAP2K	*Kinase*	*10*	*10*
MAPK	*Kinase*	*20*	*13*
PP2C	*Phosphatase*	*76*	*10*
PTP	*Phosphatase*	*28*	*1*
Total		*224*	*75 (33%)*

The categories and numbers of genes listed for each gene family were obtained from the TAIR website (TAIR, 2011). The homozygous lines utilized were available through the SALK Institute homozygous collection (Alonso et al., 2003)

Table 2. List of mutants used in this project and primers for genotyping

At number	Gene name	T-DNA allele	Primer F(5'-3')	Primer R(5'-3')
MAP4Ks				
At1g53165	*MAP4Kα1*	SK_033601	GCAATGGAGCCTCATCAGATA	ACCTGGCCCGAGTTATCATTA
At3g15220	*MAP4Kα2*	SK_116301	TCTGTCGATCTCTTTGGTGCT	CTCTTGTAAACCCGCAACATC
At1g69220	*MAP4K3*	SK_051369	GTTCCGTCTTTGTTTGCACTC	GGACACCTACCTTCCCATCAT
At5g14720	*MAP4K4*	SK_065417	AGGAAGCTGATCTCCTAATGC	AGCCTCTGCATCATAGCACAT
At1g70430	*MAP4K7*	SK_108286	GGTCACAGACTATGCCTTTGG	AATTACCTGCTCATTTCCTGG
At1g79640	*MAP4K8*	SK_067866	GTTGGAACACCTTGCTGGTAA	GCGATCATCTGCTTGAAAGAC
At1g23700	*MAP4K9*	SK_151746	ATTTCTTGATGGCCTCTCTCC	AGGGTTCACAAGAATAGCAGG
MAP3Ks				
At4g08500	*MEKK1*	SK_052557	CTCTTCGCCGTCAGGTTTTG	ACTGGTGGTCTTAGCCCCTTA
At3g13530	*Epsilon1*	SK_061724	AGCTTTCGTACGCAGTTTTGA	CAAGGGTGAGAGAGCAGTGTC
At3g07980	*Epsilon2*	SK_084747	CCAGAGTCGTTGGTGACTGTT	TATGGAATTTTGACCTATACAG
At1g09000	*MAP3K1*	SK_033751	CCGACCGATGAATCAAAATTA	TGAGCCTACTTAGCACCAACA
At1g54960	*MAP3K2*	SK_149973	GTGAGCTTCTCGCCGTTAAA	CGTGTATGTCCGAACAACCTT
At1g63700	*MAP3K4*	SK_105078	CTCGAAGCTACATTCATGGC	GATTCTTTACTTTCCCCTCCC
At4g08470	*MAP3K10*	SK_093491	GGATATCACGTCTTGGCTGAA	TCAACCCAGCAAGAATTTGTC
At4g12020	*MAP3K11*	SK_097632	AGTGGGAGTTCGACACACATC	GAAGCACGAAGATGGCTAATG
At3g06030	*MAP3K12*	SK_081990	TATCTTCTTCTCCCCATCTCC	GTTATTGGGTTTCCACATTCC
At5g55090	*MAP3K15*	SK_047537	TAACGACTCGCCGTTTGAAAT	TGATCAAATCGTGAAGACTCC
At4g26890	*MAP3K16*	SK_003255	TACCCTAAACACACACACTCG	CTTGTCTGAGATTCTTCGTCG
At5g03730	*Raf1*	*ctr1-1 EMS*	*Phenotyping: triple response of seedling*	
At1g08720	*Raf2*	SK_127158	GCGACGTGTCACAATTACCTT	CTCGATAACCTCTGGGCTACC
At1g73660	*Raf5*	SK_001982	TGGGAGAACGTATTGGACTTG	ATGCGCATCATGAATAAGGAG
At3g06620	*Raf7*	SK_000887	ATTGAAGGCGCAAGAAGAAGG	AGCGCTGCTTGTACTGTTTGC
At3g06630	*Raf8*	SK_120720	TAACAGTGCCTGTGGGGATAG	GAATGGACTGGCAACGAGATA
At3g06640	*Raf9*	SK_022298	TTGTATCGAAACTTGGCCTTG	GAATAGCGGTTGATCTGGTGA
At1g67890	*Raf11*	SK_139302	CGTTCTGCTGTTTATGGGAGC	ATGCTTGATGCGAGAAAGACC
At2g31010	*Raf13*	SK_051358	TCAATGTTGAGCACTGGTAGG	AGACCAGACACCTGAGAAAGC
At2g42630	*Raf14*	SK_137974	AATGTGCAGTGGTAACTCAGG	ACTAGGTCATGGGAATCAAGC
At1g04700	*Raf16*	SK_005607	AAGCAGAAGCATCCAGAAGTG	AAACCCCAACCTGACACACAC
At1g14000	*Raf17*	SK_101306	ACAAATCCAACATGATCCCTT	GCTTCCTAGCTCCTTCTGCAT
At1g16270	*Raf18*	SK_018487	GTTTTTGGAGTTTTCTGGTCG	GAAGTATTTTCCCACCAAAGC

Table 2. List of mutants used in this project and primers for genotyping.(continued)

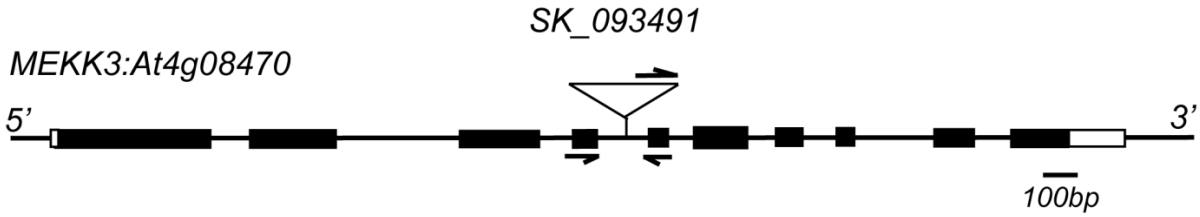
At number	Gene name	T-DNA allele	Primer F(5'-3')	Primer R(5'-3')
MAP3Ks				
At1g79570	*Raf20*	SK_143032	GAATCCTCTGCAACAGTCACC	CAGCCGGATTTTGTACTGTGT
At2g24360	*Raf22*	SK_120808	CATGGACATAAGCCATACCCC	GGTCTTTGAGAGCGAAAGCTG
At2g35050	*Raf24*	SK_107170	ATGTTCTGCTCAGCAACAGGT	ACTTTGCTGCTGCTTCCACTA
At4g35780	*Raf29*	SK_032525	CTGAATGTCAAACCATGTGC	TCAAGAGCGAGTTTGATAACG
At4g38470	*Raf30*	SK_112195	ACAAAGATCATGCTCAAGACG	GTGGAATATGGAGACATGACG
At5g50180	*Raf34*	SK_119787	GCCTCCACATCTGTGTATCGT	GCACAATCCCGTAGCTGAATA
At3g22750	*Raf39*	SK_027735	CTTGCAAGGAGCCTGTAATGG	GCAAGAATCCGTTGTGGAATA
At3g27560	*Raf41*	SK_145390	CTCCAAAATTCAATGTGAAGG	TTAAAGTCGTGGTTCAACTCG
At3g46920	*Raf42*	SK_121884	TATAGAAACCAAACAGTGGCG	AAAGATCAAATGCAGAGAGGC
At3g46930	*Raf43*	SK_022462	GAGGTCAGCAGTTCGATTCAG	AGTGTCAACGTAGCACTGAGG
At3g50720	*Raf44*	SK_103601	TGTTTTGGCTTGGTTTTTGTG	TAAACTCCAAAAGCTCGACCG
At3g50730	*Raf45*	SK_139852	TTGACTTTGAAGAGAAGGTGG	CTTGCTCACAAACAAAACACC
At3g59830	*Raf46*	SK_130077	TAGTAACAAAACCATGTCATGC	GGTTCTATGCAAGCTCCAACA
At2g17700	*Raf21*	SK_050354	AATCATGGACAACATTGCCG	GCCATCAACTTTTCGGACTTG
At4g08480	*MEKK2*	SK_150039	GCTGGCTGTTCTACAGGATCA	CAGAGGGTTGGAGATCTTGTG
At1g54510	*NEK1*	SK_065629	CGATTATACGCATCAACCTAC	GAGCGACGATGATTGGTAAAG
At3g04810	*NEK2*	SK_062288	GTTCCGTCTTTGTTTGCACTC	GGACACCTACCTTCCCATCAT
At5g28290	*NEK3*	SK_123055	AGGAAGCTGATCTCCTAATGC	AGCCTCTGCATCATAGCACAT
At3g44200	*NEK5*	SK_152782	GGTCACAGACTATGCCTTTGG	AATTACCTGCTCATTTCCTGG
At3g12200	*NEK7*	SK_134382	GTTGGAACACCTTGCTGGTAA	GCGATCATCTGCTTGAAAGAC
At3g51630	*ZIK1*	SK_039004	ATTTCTTGATGGCCTCTCTCC	AGGGTTCACAAGAATAGCAGG
At3g22420	*ZIK3*	SK_107443	TGCACAGTTCAAACTCATACG	GGACCGGTCAAATGCTAAGTG
At5g55560	*ZIK8*	SK_102847	TATGGATGTGTGCTATCAGGG	CAACCACCTGGTTTTTCAAGA
At1g64630	*ZIK10*	SK_071328	GTTTGCAGGGTTGCTATGTGT	CCATGGAAGAGAAACTTACCC
MAP2Ks				
At4g26070	*MKK1*	SK_027645	GGATCCCTTGCAGACTTGTTA	AAGAGATTGGAAGGTGCACAA
At4g29810	*MKK2*	SL_ 511_H01	AGCCATCCCTGACTCCTATCT	CTGGATGAGAGTGTAGAGTGG
At5g40440	*MKK3*	SK_051970	CAGCTGACCAGGTTGGTAAAA	CAAATCCATCAAAGAGGGAGT
At1g51660	*MKK4*	SK_142042	AAAACCAACAAAACGACACCG	TGTATACCGTTCCACCTGCTC
At3g21220	*MKK5*	SK_047797	AAGAATTGACGGCAAAGAAGG	GTTTGCGTAAACGGTTCTTCA
At5g56580	*MKK6*	SK_084332	TTACTCCCCCGTTATTTATGC	GGAAGAGATTGGGGATTCTTG

Table 2. List of mutants used in this project and primers for genotyping.(continued)

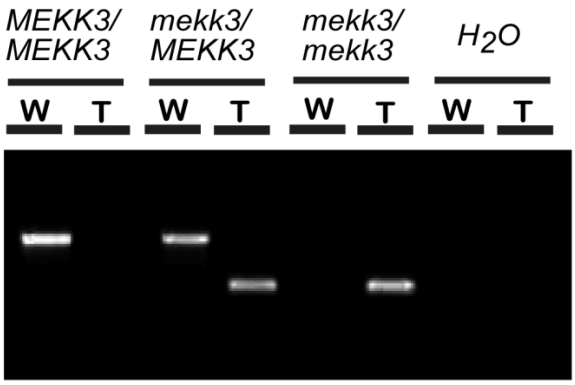
At number	Gene name	T-DNA allele	Primer F(5'-3')	Primer R(5'-3')
MAP2Ks				
At1g18350	*MKK7*	SK_009430	GTGTTTGAATGTGAAGATAAC	ATGGTGATCGAAGAGTGATGG
At3g06230	*MKK8*	SK_058723	CAATGACAACAACCAAAATGG	TCACGGAGGGAAGTAGAATCC
At1g73500	*MKK9*	SL_60_H06	GCTTATATGAGTCCGGAGAGG	GCCATTGACTTACGGTGAGAA
At1g32320	*MKK10*	SL_598_B05	AAGCGAATCGAATCGAGTTTT	CCTGAAAACCAAACCGAGACT
MAPKs				
At1g10210	*MPK1*	SK_063897	GGAACGTCGTTGGTCACTTAT	AGCAACTTTCTCGTTGGTGTC
At3g45640	*MPK3*	SK_151594	AGCACCTGAGCTTCTGTTGAA	CCGTATGTTGGATTGAGTGCT
At4g01370	*MPK4*	SK_056245	CGGTGAAACAATGACACGAGA	CCGCTTCAACAGATGGTTACG
At4g11330	*MPK5*	WI/Lox430A12	AGGAGCTACCTAAGTTCCCAAG	TGAGATGAAGGAGAAACAGAGC
At2g43790	*MPK6*	SK_073907	GGACTCTCCGTGAGATCAAGC	GAGTGGCTTACGGTCCATTAA
At1g18150	*MPK8*	SK_037501	TTCTTGGTACTCCACCTCCTG	ATCTTTCGGATCAAAGGCAAG
At3g18040	*MPK9*	SK_064439	CTGCAATCGACACACATTCAG	ATCGTTCGCCTTGATAACTTG
At1g07880	*MPK13*	SK_130193	GACTCGGATCTCGAGTTCTTG	TGCTTCAATGCTTCATCCACT
At4g36450	*MPK14*	SK_022928	GCTTGCGAGAACTTATGAACAG	GTTGGATCAAACACAAGCATC
At5g19010	*MPK16*	SK_059737	AACAGCATGCATTACCAAG	GCAGCAGCTGGATTTCTGAC
At2g01450	*MPK17*	SK_020801	AACTCGTGACTGATCTGCTTG	ACTGGACAAACCAAGATTTCAG
At1g53510	*MPK18*	SK_069399	TAATCGCAATGCAGGACTATG	TCAAGTCATCCCTGAACAAGA
At2g42880	*MPK20*	SK_090005	AGCTCATGGAATCGGATCTT	GTTATATCGCGGCAACACAC
Phosphatase				
At5g51760	*AHG1*	SK_005558	TAACACCTGAGCCTAACACGC	CATCCATCCTCTTGAAACTCC
At5g59220		SK_142672	TTCAGTCTGTGGAAGAAGACG	TCTTAATCGTCGTCCAGGTGT
At2g29380		SK_033011	TTTGATTGGACCACGAATCTC	CACACCGAAGAAACACCGTAT
At1g72770	*HAB1*	SK_002104	TAAGGTTCTTGAGGCTGTTGCG	CCGTCTCCTTGCTATTTCGCA
At1g07630	*AtPLL5*	SK_130437	CGGTACTAACCAGCAGAGTCG	GTTGTCGTCGTTGTGTCTTCC
At5g02760		SK_099356	TCGACAAGTAAGTTCAATGCC	TGGTCTACTGGTGTTCTGTGC
At3g12620		SK_036920	CCTCTTCGACAACATCAGGAG	TGTCAACAGCTTCTTGATTGG
At5g06750		SK_026300	GTTAATTCTCGTGGTCGTGGA	TCCAGAAACGAACAAACAGAG
At4g32950		SK_112797	CCTGCCACACCACACTTTAAT	CAACGACACACACCATTCCTA
At3g16800		SK_061302	CGCTGTTTTGCAGGTAACATA	ATCGGTGGCCAAGATTAGAAA
At2g04550	*IBR5*	SK_039359	GGGTTTTGCCTTGTAGATGAA	GCCTGGACTTATGTCAGTGCT

Table 2. List of mutants used in this project and primers for genotyping (continued)**.** ‘At number’ is the gene ID in the Arabidopsis genome. ‘Gene name’ is the commonly used gene name. ‘T-DNA’ allele is the identification number of the mutant allele from the SALK Institute. Primer F and Primer R are the two primers used to perform PCR-based genotyping.

(A)


SK_093491
MEKK3:At4g08470
5'
3'
100bp

(B)


MEKK3/ MEKK3
mekk3/ MEKK3
mekk3/ mekk3
H2O
W
T
W
T
W
T
W
T

(C)

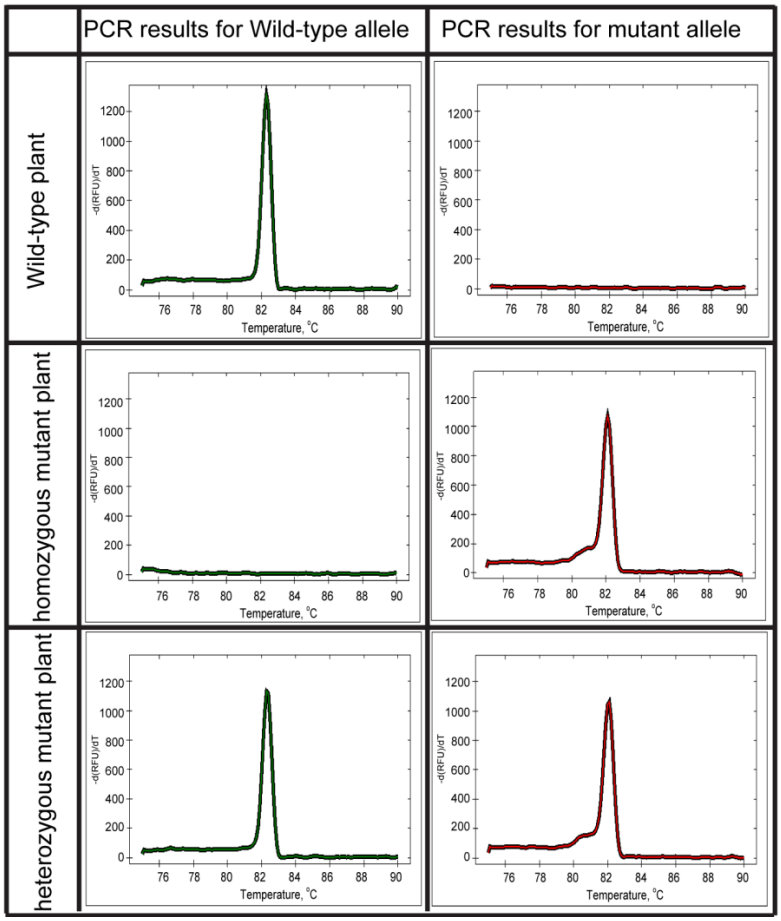
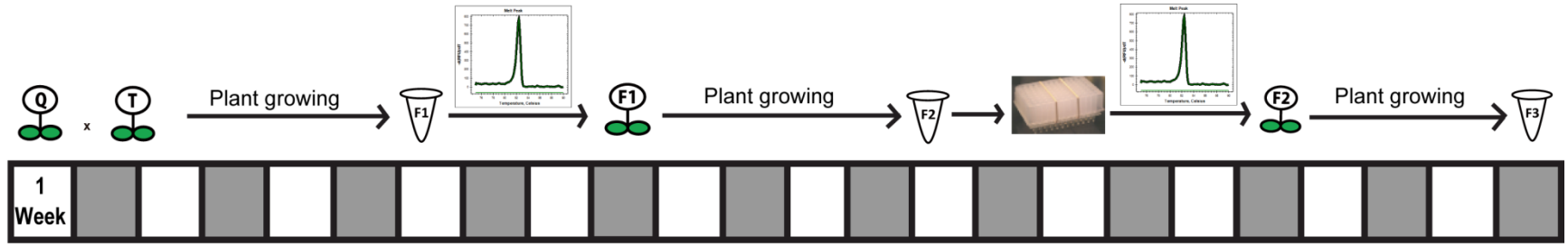

PCR results for Wild-type allele
PCR results for mutant allele
Wild-type plant
homozygous mutant plant
heterozygous mutant plant
-d(RFU)/dT
Temperature, °C

Fig. 2. PCR-based genotyping using agarose gel electrophoresis and melt-curve analysis. (A) Diagram of the *MEKK3* locus. Black boxes refer to exons and the triangle represents the T-DNA insertion position. Arrows indicate the locations of the primers used for PCR-based genotyping. (B) Electrophoresis results of PCR-based genotyping results. "W" refers to PCR products amplified from the wild-type allele and "T" refers to PCR products amplified from the T-DNA mutant allele. Corresponding melt curve results for the same PCR reactions are shown in (C). The green line represents the results obtained with PCR reactions that target the wild-type allele. The red line represents the results of PCR reactions that target the T-DNA mutant allele. The graphs present the derivative of the rate of change in fluorescence on the y-axis and the temperature on the x-axis. For this analysis PCR reactions containing the fluorescent dye EvaGreen are slowly heated and total fluorescence is measured at increments throughout the heating process. Because EvaGreen fluorescence is greatly enhanced when the dye is bound to double-stranded DNA, total fluorescence in the tube rapidly drops when the double-stranded DNA reaches its melting temperature. The transformed data presented above highlights the melting temperature of the PCR product as a sharp peak on the graph.



Plant query and target mutants and manually cross	Collect seeds from cross	Genotype F1 seedlings	Collect F2 seeds	Ice-Cap Analysis of F2 population	Collect F3 double homozygous seeds
Produce double-heterzygous F1 plants		Collect F2 seeds from F1 plants		Produce double-homozgyous F3 seeds	
Stage I- 7 weeks		**Stage II- 8 weeks**		**Stage III- 9 weeks**	

Total 6 months

Fig. 3. Timeline of the double-mutant production pipeline. The white and gray boxes each indicate a time period of one week. The timeline is divided into three stages. Stage 1 involves producing double-heterozygous mutants (F1) by manually crossing two single-mutants. Stage 2 involves propagating F1 plants and collecting F2 seed for each double-mutant combination. Stage 3 involves identifying double-homozygous mutants using the Ice-Cap system, propagating double-homozygous F2 plants, and collecting F3 seed. The total time necessary to complete one cycle of the pipeline is 6 months, and a new cycle can be started every two weeks. When fully operational, the pipeline has batches of 32 double-mutants moving through the pipeline, with each batch spaced two weeks apart. Using this strategy, one scientist can produce 32 new double-mutants per week, or 768 per year.

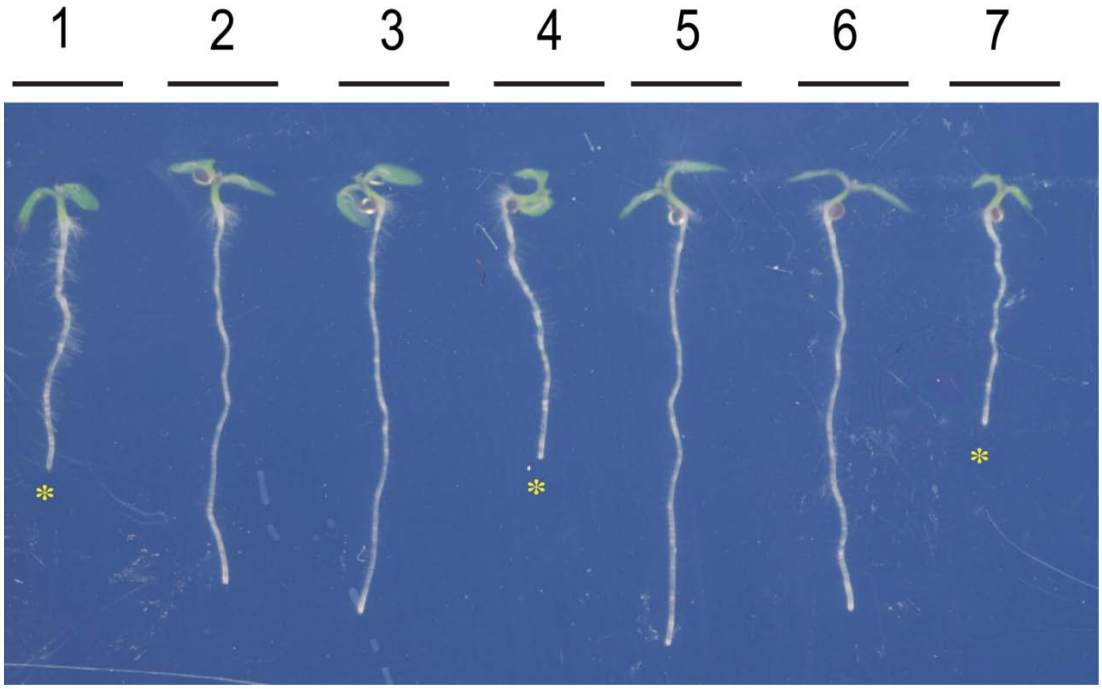

1
2
3
4
5
6
7

Fig. 4. Representative 6-day-old seedlings grown on vertically oriented agar plates from a population that is segregating the *mpk4* mutation. * indicates homozygous single-mutant *mpk4* seedlings (No. 1, 4, and 7). The *mpk4* mutant seedlings show a shorter root and smaller cotyledons than wild-type or *mpk4* heterozygous seedlings.

Table 3 List of double-mutants within the MPKs

MPKs	*mpk20*	*mpk18*	*mpk17*	*mpk16*	*mpk14*	*mpk13*	*mpk9*	*mpk8*	*mpk6*	*mpk5*	*mpk4*	*mpk3*
mpk1	SH1012	SH1011	SH1010	SH1009	SH1008	SH1007	Cross fail	SH1005	SH1004	SH1003	SH1002	SH1001
mpk3	SH1023	SH1022	Cross fail	SH1020	SH1019	SH1018	SH1017	SH1016	Lethal	SH1014	SH1013	
mpk4	SH1033	SH1032	SH1031	SH1030	SH1029	SH1028	SH1027	SH1026	SH1025	SH1024		
mpk5	SH1042	SH1041	SH1040	SH1039	SH1038	SH1037	SH1036	SH1035	SH1034			
mpk6	Linked	SH1049	SH1048	SH1047	SH1046	SH1045	SH1044	SH1043				
mpk8	SH1057	SH1056	SH1055	SH1054	SH1053	SH1052	SH1051					
mpk9	SH1063	SH1062	SH1061	SH1060	SH1059	SH1058						
mpk13	SH1068	SH1067	SH1066	SH1065	SH1064							
mpk14	SH1072	SH1071	SH1070	SH1069								
mpk16	SH1075	Sh1074	SH1073									
mpk17	SH1077	SH1076										
mpk18	SH1078											

LITERATURE CITED

Alonso et al., 2003. Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301:653-657.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature 415: 977-983.

Bush, S.M., and Krysan, P.J. (2007) Mutational evidence that the Arabidopsis MAP kinase MPK6 is involved in anther, inflorescence, and embryo development. J Exp Bot. 58:2181-91.

Byrne, A.B., Weirauch, M.T., Wong, V., Koeva, M., Dixon, S.J., Stuart, J.M., and Roy, P.J. (2007) A global analysis of genetic interactions in Caenorhabditis elegans. J Biol. 6:8.

Clark, K.A. and P.J Krysan. 2007. Protocol: An improved high-throughput method for generating tissue samples in 96-well format for plant genotyping (Ice-Cap 2.0). Plant Methods, 12:3-8.

Costanzo, M., Baryshnikova, A., Myers, C.L., Andrews, B., and Boone, C. (2011) Charting the genetic interaction map of cell. Current opinion in Biotechnology 22:66-74.

Davierwala, A.P., Haynes, J., Li, Z., Brost, R.L., Robinson, M.D., Yu, L., Mnaimneh, S., Ding, H., Zhu, H., Chen, Y., Cheng, X., Brown, G.W., Boone, C., Andrews, B.J., and

Hughes, T.R. (2005) The synthetic genetic interaction spectrum of essential genes. Nature genetics 37:1147-1152.

He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nürnberger, T., and Sheen, J. (2006) Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. Cell 125:563-75.

Kermekchiev, M.B., Tzekov, A., and Barnes, W.M. (2003) Cold-sensitive mutants of Taq DNA polymerase provide a hot start for PCR. Nucleic Acids Res. 31:6139-6147.

Kosetsu, K.K., Matsunage, S., Nakagami, H., Colcombet, J., Sasabe, M., Soyano, T., Takahashi, Y., and Machida, Y. (2010) The MAP kinase MPK4 is required for Cytokinesis in Arabidopsis thaliana. Plant Cell 22: 3778-3790.

Krysan PJ (2004) Ice-cap. A high-throughput method for capturing plant tissue samples for genotype analysis. Plant Physiol 135: 1162-1169.

Krysan, P.J., Jester, P.J., Gottwald, J.R., and Sussman, M.R. (2002) An Arabidopsis mitogen- activated protein kinase kinase kinase gene family encodes essential positive regulators of cytokinesis. Plant Cell 14:1109-1120.

Lampard, G.R., MacAlister, C.A., and Bergmann, D.C. (2008) Arabidopsis Stomatal initiation is controlled by MAPK-Mediated regulation of the bHLH SPEECHLESS. Science 322:1113-1116.

Lee, J.S., Huh, K.W., Bhargava, A., and Ellis, B.E. (2008) Comprehensive analysis of protein-protein interactions between Arabidopsis MAPKs and MAPK kinases helps define potential MAPK signalling modules. Plant Signaling & Behavior 3:1037-1041

Lin et al., 2008. A comprehensive synthetic genetic interaction network governing yeast histone acetylation and deacetylation. Genes & Development, 22:2062-2074.

Menges, M., Dóczi, R., Okrész, L., Morandini, P., Mizzi, L., Soloviev, M., Murray, J.A., and Bögre, L. (2008) Comprehensive gene expression atlas for the Arabidopsis MAP kinase signalling pathways. New. Phytol. 179:643-62.

Mészáros, T., Helfer, A., Hatzimasoura, E., Magyar, Z., Serazetdinova, L., Rios, G., Bardóczy, V., Teige, M., Koncz, C., Peck, S., and Bögre, L. (2006) The Arabidopsis MAP kinase kinase MKK1 participates in defence responses to the bacterial elicitor flagellin. Plant J. 48, 485–498.

Nakagami, H., Soukupova, H., Schikora, A., Zarsky, V. and Hirt, H. (2006) A Mitogen-activated protein kinase kinase kinase mediates reactive oxygen species homeostasis in Arabidopsis. J. Biol. Chem. 281, 38697–38704.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J. (2000) Arabidopsis MAP Kinase 4 negatively regulates systemic acquired resistance. Cell 103: 1111-1120.

Popescu, S.C., Popescu, G.V., Bachan, S., Zhang, Z., Gerstein, M., Snyder, M., and Dinesh-Kumar, S.P. (2009) MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. Genes Dev 23: 80-92.

Su, S., Clark, K.A., Gibbs, N.M., Bush, S.M., Krysan, P.J. Ice-Cap: A Method for Growing Arabidopsis and Tomato Plants in 96-well Plates for High-Throughput Genotyping. J. Vis. Exp. (57):e3280

Tong, A.H., Lesage, G., Bader, G.D., Ding, H., Xu, H., Xin, X., Young, J., Berriz, G.F., Brost, R.L., Chang, M., Chen, Y., Cheng, X., Chua, G., Friesen, H., Goldberg, D.S., Haynes, J., Humphries, C., He, G., Hussein, S., Ke L, Krogan, N., Li, Z., Levinson, J.N., Lu, H., Ménard, P., Munyana C, Parsons, A.B., Ryan, O., Tonikian, R., Roberts, T., Sdicu, .M., Shapiro, J., Sheikh, B., Suter, B., Wong, S.L., Zhang, L.V., Zhu, H., Burd, C.G., Munro, S., Sander, C., Rine, J., Greenblatt, J., Peter, M., Bretscher, A., Bell, G., Roth, F.P., Brown, G.W., Andrews, B., Bussey, H., and Boone, C. (2004) Global mapping of the yeast genetic interaction network. Science 303:808-813.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C., Zhang, S. (2007) Stomatal Development and Patterning Are Regulated by Environmentally Responsive Mitogen-Activated Protein Kinases in Arabidopsis. Plant Cell 19: 63-73.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999) Mitogen-Activated Protein Kinase: Conservation of a Three-Kinase Module From Yeast to Human. Physiol Rev. 79: 143-180

Chapter 4

Genetic interaction analysis of genes related to MAP kinase pathways in Arabidopsis

ABSTRACT

In the previous chapter, I described how I produced 287 double-mutants using my high-throughput double-mutant generating pipeline. Here, I performed qualitative and quantitative phenotype assays to identify evidence of genetic interactions using these double-mutants. Through qualitative phenotype observation, I identified two double-mutants in which the dwarf phenotype associated with the *mpk4* single-mutant was rescued: *mekk2;mpk4* and *mkk1;mpk4*. I also measured root growth rate as a quantitative phenotype to search for evidence of interactions. From this quantitative screen I identified five gene pairs that showed reproducible evidence of genetic interaction following two rounds of independent testing. These five double-mutants are *mpk4;anp2, mpk4;anp3, mpk4;mpk5, mpk4;mpk13,* and *mpk4;mpk18*. To summarize, using the high-throughput double-mutant pipeline that I developed, I was able to find seven double-mutant combinations that demonstrate genetic interaction. I also confirmed two previously reported genetic interactions as a result of my systematic screen. Taken together, I observed evidence for genetic interaction in 9 out of the 287 double-mutants that I produced, corresponding to a rate of 3.1%.

INTRODUCTION

In Chapter 3, I described the 289 double-mutants that I constructed for this project. Two of these double-mutants, *mekk1*;*mpk4* and *mpk3*;*mpk6*, show synthetic lethality as was reported previously (chapter 2; Wang et al., 2007). In this chapter I will discuss the phenotypic analyses that I performed on the remaining 287 viable double-mutants in order to search for evidence of genetic interactions.

With a genetic interaction screen, the object of performing phenotypic assays is to search for situations where the phenotype of the double-mutant cannot be explained by the phenotypes of the two single-mutants. With a multicellular organism such as Arabidopsis, the list of possible phenotypic screens that could be performed is extensive. My objective for this project was not to exhaustively catalog all the phenotypic properties of my collection of double- and single-mutant lines. Rather, I was interested in performing a pilot study to provide me with an estimate of the extent to which evidence of genetic interactions could be detected using the type of double-mutant collection that I produced for this study. I chose to use root growth rate as the quantitative phenotype for my study because this feature of Arabidopsis growth lends itself well to large-scale quantitative analysis. It seems likely that if one were to

perform a large number of distinct phenotypic measurements on this collection of single- and double-mutant lines that one would find additional instances supporting genetic interaction between pairs of genes that have not emerged from the initial phenotypic testing that I report in this chapter. Again, my objective was not to exhaustively screen this collection of mutant lines under a large battery of conditions. Instead I wanted to provide a first-estimate of the rate with which evidence of genetic interaction could be observed with my collection of mutant lines in order to determine if this approach constitutes a productive approach to exploring gene function.

There is evidence in the literature that performing quantitative phenotyping is an effective way to increase the sensitivity with which evidence of genetic interaction can be observed. For example, by performing quantitative phenotype assessment with the large-scale genetic interaction analyses in *C. elegans*, the rate of identification of genetic interactions increased from 0.5% to 13% (Lehner et al., 2006; Byrne et al., 2007).

In addition to quantitative measurement of root growth, I also performed visual screening of general plant development of the mutant lines in my collection to search for obvious developmental differences. This approach led to the identification of two instances of genetic interaction in my collection. In this chapter, I will provide details

of the phenotypic assays used in my large-scale screen, and I will also describe the candidate gene pairs showing evidence of genetic interaction based on these phenotypic assays.

RESULTS

Quantitative analysis of root growth rate.

I generated 287 double-mutant lines for this study. In order to search for evidence of genetic interaction within this collection, root growth rate was measured for all of the single- and double-mutant lines in my collection. Seeds from each double-mutant line were sown on agar plates and grown in a vertical orientation, as were seeds from the two corresponding single-mutants and wild-type Columbia-0 (Col-0). Root tip positions of 4-day-old seedlings were marked and subsequently images of 6-day-old seedlings were scanned. The difference in root length between days four and six was determined by measurement of the images using ImageJ software (Rasband, 1997-2011). This measurement was converted to the rate of root growth expressed in centimeters per day.

In order to identify pairs of genes that show evidence of genetic interaction, I determined whether the root growth rate in a double-mutant showed a statistically significant difference from the root growth rate in either of the two single-mutant parents. To analyze the quantitative root growth data, I used an unpaired t-test with a significance cut-off of $P<0.001$. Figure 1 shows representative results of double-mutants that do not show the evidence of a genetic interaction. Based on the t-test, 66 out of the 287 double-mutants that I analyzed were candidates for genetic interaction. At this stage of my project, it was impractical to re-test and follow up all 66 of these double-mutants. Therefore, I chose a second criterion based on the variance of the data to identify candidates on which to perform follow-up analyses. The mean rate of root growth was graphed for each double-mutant as well as the two corresponding single-mutants and wild-type Col-0. Standard deviation was calculated and represented as error bars in these graphs. Double-mutant lines for which the error bars did not over-lap with the error bars of either of the single-mutants were selected. In this step, I narrowed down my list of candidates from 66 double-mutants down to 26 double-mutants. For each of these 26 candidates, a second, independent root growth rate experiment was performed. The quantitative data from the second round were also analyzed by t-test, P value <0.001, to determine

if the difference between the double-mutants and their two corresponding single-mutants was reproducible. Five out of these 26 double-mutants showed reproducible differences in the rate of root growth in the two independent trials, and these five lines were chosen for follow-up experiments. Of these five genetic interactions, two of them are from query-target pair screening and three are interactions within the *MPKs*. Figure 1 shows an overview of the results obtained in the root growth rate experiments, and figure 3 shows the root growth results of these five putative interaction pairs.

Genetic interaction candidates identified in the root growth rate experiments

Query-target pairs. I identified two MAP3K genes, *ANP2* and *ANP3* that show evidence of genetic interaction with *MPK4* based on measurement of root growth rate. The full name of the *ANP* genes is *Arabidopsis homologs of Nucleus- and Phragmoplast- localized kinase.* In previous research, these two genes have been shown to be involved in cytokinesis, and *anp2;anp3* double-homozygous-mutants show slow growth, dwarfism, and dark green leaves in the Ws background (Krysan et al., 2002). In my screen, the single-mutant lines of *ANP2* and *ANP3* in the Col-0 background are used. The gene locations of the T-DNA insertions for these

single-mutants are shown in figure 4. The *anp2* T-DNA insert is in the second intron, and *anp3* T-DNA insertion is in the 11th exon. *anp2;anp3* double-mutants display the same dwarfism in the Col-0 background that was previously described for *anp2;anp3* plants in the Ws background (data not shown).

The root growth rates of *anp2*;*mpk4* and *anp3*;*mpk4* show 35% and 46% reduction respectively, compared to *mpk4*. Figure 5 shows representative 6-day-old seedlings of *anp2;mpk4* and *anp3;mpk4* double-mutants, as well as the corresponding single-mutants and wild-type. Neither the *anp2* nor the *anp3* single-mutants show a reduced root growth rate when compared to wild-type Col-0. These results suggest that the abnormal phenotype observed in the double-mutants is caused by genetic interaction of the two *ANP* genes with *MPK4* rather than an additive phenotype of the corresponding single-mutants.

MPK-MPK pairs. Three novel genetic interactions were identified from my double-mutant screening within the MPK gene family: *MPK4-MPK5*, *MPK4-MPK13* and *MPK4-MPK18.* Based on a phylogenetic tree of the MPK genes (Fig. 6), *MPK5* and *MPK13* belong to the B group of *MPKs,* which is the same subgroup as *MPK4*, while *MPK18* belongs to the more distantly related D group (MAPK Group, 2000). There are no previous studies establishing the gene functions of *MPK5* and *MPK13* in

Arabidopsis. *MPK18* has been reported to be involved in microtubule stabilization (Walia et al., 2009), a process in which *MPK4* has also been implicated (Beck 2010 organization).

The locations of the T-DNA insertion in each of these single-mutants are given in figure 4. Each of these three mutant lines was analyzed and it was determined that no full length mRNA was detected and no chromosomal translocations were associated with the T-DNA insertions (Clark, 2010). Figure 7 displays representative 6-day-old seedlings of the *mpk4;mpk5, mpk4;mpk13*, and *mpk4;mpk18* double-mutants, as well as the corresponding single-mutants and wild-type seedlings. Double-mutants of *mpk4;mpk5* show a 33% reduction of root length when compared to the *mpk4* single-mutants. *mpk5* single-mutants have a root length that is similar to wild-type seedlings. The same tendency was also observed in the *mpk4;mpk13* and *mpk4;mpk18* double-mutants. These double-mutants show 34% and 24% root growth reduction, respectively, compared to *mpk4* single-mutants.

Genetic interaction candidates identified via qualitative phenotype analysis

As an alternative strategy to obtain evidence of genetic interaction, qualitative phenotype observation was also performed for all 287 double-mutants. 6-day-old

seedlings from agar plates were transferred into soil and grown to maturity in under constant light in a growth chamber. Each week, phenotypic observation was completed for each plant on various features including overall plant size, rosette leaf shape, fertility, and petal abscission. For qualitative phenotype observation, very obvious differences between double-mutants and their corresponding two single-mutants were of interest. From this analysis, I found evidence of genetic interaction for two gene pairs: *MEKK2-MPK4* and *MKK1-MPK4.*

MEKK2 and MPK4. As mentioned, *mpk4* single-mutants display a strong dwarf phenotype caused by constitutive activation of pathogen defense pathways (Petterson et al., 2000). In my screen, I found that the *mekk2* mutant is able to dramatically rescue the dwarf phenotype of *mpk4*. Leaf size and morphology of *mekk2*;*mpk4* double-homozygous mutant plants are similar to wild-type, which is in strong contrast to the *mpk4* single-homozygous mutant (Fig. 8).

MEKK2 is a MAP3K gene that belongs to the MEKK subgroup (MAPK Group, 2002). The location of the T-DNA insertion in the *mekk2* mutant allele is ca. 300bp upstream of the first codon of the gene's open reading frame (Fig. 4). *MEKK2* and the two most similar genes in the genome, *MEKK1* and *MEKK3,* form a tandemly duplicated group of genes on chromosome 4 in Arabidopsis. Of these three genes,

MEKK1 is the only one for which an abnormal phenotype has been reported as a result of gene inactivation (Suarez-Rodriguez et al., 2007). *mekk1* single-mutant plants show a severe dwarf phenotype that is similar to that of *mpk4* mutant plants. Although *MEKK2* is closely related to *MEKK1* in Arabidopsis, there is no obvious abnormal phenotype observed in the *mekk2* single-homozygous mutant plants when they are grown under laboratory condition (Fig. 8).

The other interesting feature of the *mekk2*;*mpk4* double-mutant is that, although the *mekk2* mutant background can dramatically rescue the dwarfism caused by *mpk4*, there is no significant change observed in the root growth deficiency displayed by *mpk4* single-mutants (Fig. 9). In other words, the mutation of *MEKK2* gene rescues the dwarfed shoot phenotype of *mpk4*, but cannot rescue the abnormal root growth phenotype of *mpk4*.

*MKK1 and MPK4***.** I also identified a second mutation that was able to rescue the *mpk4* dwarf phenotype. Mutation of *MKK1*, a member of the MAP2K gene family, is able to partially rescue the dwarfism present in *mpk4* single-mutant lines (Fig. 10). The T-DNA insertion of *mkk1* is located in intron 5 (Fig. 4). This mutant line has been reported as a loss-of-function mutant in previous studies (Meszaros et al., 2006). According to several reports, *MKK1* and its homologue *MKK2* act in a functionally

redundant manner upstream of *MPK4* to regulate pathogen resistance (Gao et al., 2008; Qiu et al., 2008). *mkk1;mkk2* double-mutant plants show severe dwarfism which is similar to that displayed by *mpk4* single-mutant plants (Qiu et al., 2008). Contrary to the idea that these two genes are functionally redundant, I observed that the phenotypes of *mkk1;mpk4* and *mkk2;mpk4* double-homozygous mutants are different from each other. As shown in figure 10(A-I), *mkk1;mpk4* plants show a partially rescued dwarf phenotype when compared to *mpk4* single-mutants, while *mkk2;mpk4* plants are similar in appearance to the *mpk4* single-mutants.

An additional aspect of the phenotypic rescue observed in *mkk1;mpk4* double-homozygous mutant plants can be observed when the plants initiate flowering. In *mpk4* single-mutant plants, the primary flower bolt does not elongate and also does not produce siliques (Fig. 10K-L). In *mkk1;mpk4* double-mutant plants, the primary bolt can extend and can produce small siliques and a few shrunken seeds (Fig 10J). By contrast, *mkk2* does not rescue any of the abnormal *mpk4* phenotypes.

When root growth rate was measured for these two double-mutant lines, neither *mkk1*;*mpk4* nor *mkk2*;*mpk4* showed any evidence of a phenotypic difference compared to the *mpk4* single-mutant line. This result indicated that *mkk1* is not able to rescue the root growth defects seen in *mpk4* single-mutant plants.

DISCUSSION

Based on the phenotypic screening that I performed, I found evidence of genetic interaction for a total of nine gene pairs, including seven novel interactions found in this study and two synthetic lethal pairs that have been previously reported (Table 1). Among the seven new interaction pairs, five were identified via the quantitative analysis of root growth rate and two were found using qualitative phenotypic observation. All of the genetic interactions identified using the quantitative analysis were negative interactions that reduced root growth rate. The two interactions from qualitative observation were positive interactions that rescued the *mpk4* dwarfism phenotype. To summarize, I screened a total of 289 gene pairs and found nine pairs that displayed evidence of genetic interactions. The rate of identifying evidence of genetic interaction in my screen was therefore 3.1%. I would expect that if I performed additional quantitative phenotypic assays on other aspects of Arabidopsis growth or under different growth conditions that additional evidence of genetic interactions could be identified.

MPK4 plays multiple roles in different signaling pathways

Of the nine pairs of genes for which I found evidence of genetic interaction, eight of these pairs involved *MPK4*. With the exception of the *mekk1;mpk4* double-mutant which shows synthetic lethality, the phenotypes I observed can be divided into two categories: (1) synthetic enhancement of defects in root growth rate and (2) synthetic rescue of the dwarf phenotype. Based on previous studies, the dwarfism of *mpk4* single-mutants is caused by constitutive activation of pathogen defense responses (Peterson et al., 2000). The abnormal root phenotype of *mpk4* single-mutants has been suggested to be due to defects in cytokinesis (Kosetsu et al., 2011). Interestingly, the two mutants that rescue the *mpk4* dwarf phenotype, *mekk2* and *mkk1,* did not rescue the *mpk4* abnormal root phenotype. This result may suggest that the biological processes related to these two abnormal features of *mpk4* mutant plants are regulated independently. The abnormal phenotype of the *mpk4* single-mutant plants may therefore be a compound phenotype caused by the disruption of multiple, independent biological processes. The selective rescue of some, but not all, of the *mpk4* mutant phenotypes by *mekk2* and *mkk1* could provide geneticists with useful tools for more carefully dissecting the different pathways in which *MPK4* functions. This situation highlights one of the potential benefits of performing systematic genetic interaction screening in that it has the potential to provide both additional insights into a

signaling pathway as well as new genetic reagents for more precise study of those pathways.

Systematic analysis of genetic interactions can provide new information about the functional relationship between homologous genes

Compared to studying gene function in single-mutants, using double-mutants to study genetic interaction can provide additional information about the functional relationship between homologous genes. For example, *ANP2* and *ANP3* genes were previously known to be functionally redundant because *anp2;anp3* double-homozygous mutants show a cytokinesis deficiency that cannot be observed in either single-mutant (Krysan et al., 2002). If *ANP2* and *ANP3* work redundantly upstream of *MPK4*, one would expect *anp2*;*mpk4* and *anp3*;*mpk4* double-mutants to show the same mutant phenotype as the *mpk4* single-mutant because *ANP2* and *ANP3* should be able to compensate for the absence of the other if their functions are truly redundant. However, our results show that *anp2;mpk4* and *anp3;mpk4* double-homozygous mutants both show a reduction of root growth rate compared to *mpk4*, which conflicts with the idea of complete redundancy of *ANP2* and *ANP3*. These results suggest that the relationship between *ANP2* and *ANP3* is more

complicated than simple functional redundancy upstream of *MPK4* in the cytokinesis pathway and that each may have a unique function.

In addition to the redundancy described for *ANP2* and *ANP3*, the gene pair *MKK1* and *MKK2* has also been reported to be functionally redundant. *MKK1* and *MKK2* work upstream of *MPK4* to control innate immunity in Arabidopsis, and the *mkk1;mkk2* double-homozygous mutants show the same abnormal phenotype as *mpk4* single-mutant (Gao et al., 2008). However, when I produced double-homozygous mutant *mkk1;mpk4* and *mkk2;mpk4*, I found that the *mkk1* background can partially rescue *mpk4* dwarf phenotype. This result is not observed in the *mkk2* background. In other words, *mkk1* and *mkk2* behave differently in the *mpk4* background. Moreover, if *MKK1* acts upstream of *MPK4* in a simple linear pathway, the double-mutant of *mkk1;mpk4* should show the same phenotype as the *mpk4* single-mutant due to epistasis. However, our results show that *mkk1* single-mutant partially rescues the *mpk4* dwarfism. This unexpected result shows that MKK1 does not act exclusively upstream of MPK4 in a linear signaling pathway. Also, it provides evidence that the functional relationship between *MKK1, MKK2*, and *MPK4* is more complicated than the previously suggested model.

In the cases of *ANP2/3* and *MKK1/2*, each gene in the pair appears to have unique roles outside of the redundant functions that have been previously suggested. Loss-of-function of these unique roles are not detected under standard conditions. When each of these mutant genes is paired with the *mpk4* mutant, however, *mpk4* provides a sensitized background that allows us to identify emergent phenotypes that may be related to the unique function of each of these genes. Biochemical studies have been reported that describe various treatments that activate the MKK1 protein alone or the MKK2 protein alone, such as H_2O_2, the bacterial elicitor flagellin, cold stress, and salt stress. (Teige et al., 2004; Qiu et al., 2008; look at Xing et al., 2008).

Genetic interaction screening identifies functional relationships within the MAP kinase gene family.

As mentioned in chapter 1, there are 20 members in the MAP kinase (MPK) gene family, and there are four subgroups of MAP kinases in Arabidopsis. Based on my genetic interaction screening within the *MPK*s, I identified three gene pairs showing evidence of genetic interaction that had not been previously described: *MPK4-MPK5*, *MPK4-MPK13,* and *MPK4-MPK18.* *MPK5* and *MPK13* both belong to the B group of *MPK*s, the same group as *MPK4*. There is no previous study indicating any possible

function of these two genes, and *mpk5* and *mpk13* single homozygous mutant plants appear phenotypically wild-type. However, based on biochemical evidence reported previously, there are some clues for us to predict the pathways and functional relationship between these genes. First, there are two separate studies of kinase activity showing that the constitutively activated MAP2K MKK6 can activate MPK5 and MPK13 in vitro (Kosetsu et al., 2010; Melikant et al., 2004). These results show that MKK6 may act upstream of MPK5 and MPK13. Interestingly, there is another report showing that *MKK6* interacts with *MPK4* genetically and biochemically and acts upstream of *MPK4* to regulate cytokinesis (Kosetsu et al., 2010). To combine the results from previous studies with the genetic interaction results reported here, *MPK5* and *MPK13* may also be involved in the *MKK6-MPK4* pathway.

The other candidate identified as interacting with *MPK4* is *MPK18*, which belongs to the D group of MPKs. Based on a previous report, *MPK18* may be involved in stabilizing microtubules: *mpk18* shows defects in microtubule-related functions and also shows moderately hyperstabilized microtubules (Walia et al., 2009). *mpk4* single-mutants show short roots because of their deficiency in cell structure (Beck et al., 2010), while the roots of *mpk18* plants do not appear different from those of wild-type. One explanation for these results is that both MPK4 and MPK18 are involved in the

regulation of the microtubule organization process. Double-mutants of these two genes interfere with this process more severely than disruption of either gene on its own, thus causing the more severe root growth defects seen in the double-mutant.

Genetic interaction network of MAPK related gene in Arabidopsis

To organize my genetic interaction results, I combined the interactions identified from this screen with interactions described in the literature to produce a map of the genetic interaction network for MAPK related genes in Arabidopsis (Fig. 11). Based on this map, *MPK4* is a hub gene in the network because it interacts with multiple other genes. As mentioned in chapter 1, work with yeast has suggested that hub genes are more likely to play a critical role for the functioning of the organism. By knocking out a hub gene, one may have a higher chance to observe a serious mutant phenotype because of disrupting the multiple functions it controls (Dixon et al., 2009). This model is consistent with our results because we observed the most interactions with *MPK4* and *mpk4* single-mutant plants display a severe mutant phenotype.

Based on the gene functions reported in previous studies, the MAPK related genes in my network can be divided into two major groups. One contains the genes that have been shown to be related to pathogen defense. The other group consists of the

genes that are involved in cytokinesis or microtubule organization. As with other genomic-style screening projects, the object of the work reported in this thesis was to provide novel information about gene function that may not be available via existing methodologies. As reported here, I determined new functional relationships between a number of MAPK-related genes as a result of my screening project. The information produced by my screening project should provide the starting point for follow-up studies that aim to explore in more detail the nature of the functional associations between the gene pairs identified as displaying genetic interaction. Genetic interaction studies provide a starting point for exploring in more detail the underlying signaling networks that operate within a living organism. The interaction pairs identified in this project should provide the starting material for exploring signaling pathways in Arabidopsis that make use of the MPK4 protein, including pathways regulating cell division and pathogen response.

MATERIAL and METHODS

Plant material and growth condition

Plant material and growth conditions used in this chapter are the same as those listed in Chapter 3. *Arabidopsis thaliana* plants were grown in soil under continuous light at 18-20^{0}C. Plant materials used in this work is all in the Columbia-0 (Col-0) ecotype. 88 T-DNA insertion single-mutants (Chapter 3, Table 2) were used in this work and all the lines were ordered from ABRC stock center (http://abrc.osu.edu/; Alonso et al., 2003). Single-mutants were tested by PCR-based genotyping to confirm the T-DNA insertion location and genotype. For mutant allele information, please see Material and Methods section in chapter 3 for detail. All the primers used for genotyping are also listed in Chapter 3, Table 2.

Quantitative analysis of root growth rate.

For quantitative analysis, double-mutant (F3) seeds were plated on 1% agar plates with 0.5x Murashige and Skoog media. After cold treatment for at least 48 hours to stratify the seeds, the plates were grown vertically under continuous light at 18-20^{o}C. Root tip positions of 4-day-old seedlings were marked and subsequently images of 6-day-old seedlings were scanned at resolution of 600dpi by an Epson v700 scanner. The root length was measured using ImageJ software from NIH image group (Rasband, 1997-2011, http://imagej.nih.gov/ij/). The difference between 4-day and 6-day root

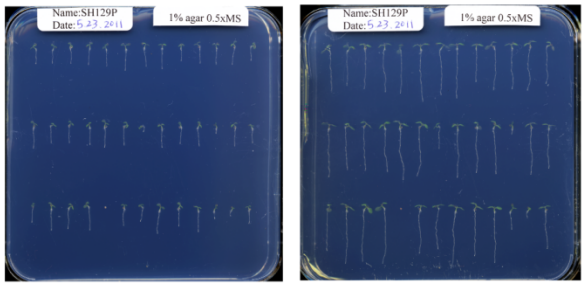
length was calculated and used as the final index to determine the evidence of genetic interaction.

Qualitative phenotype analysis.

6-day-old seedlings from the quantitative analysis were transferred to soil and grown under continuous light at 18-20^{0}C. Gross phenotypic observations were complete each week until plant flowering was completed.

287 double-mutants

Each double mutant, its corresponding single mutants, and wild-type are grown on vertically oriented agar plates. Root growth rate between day 4 and day 6 is determined.



4-day-old seedlings 6-day-old seedlings

t-test, $P < 0.001$
Each double-mutant compared to its two single-mutants

66 double-mutants

Select double-mutants for which error bars do not overlap with corresponding single-mutants

26 double-mutants

Repeat root growth experiments
t-test, $P<0.001$

5 genetic interaction candidates

Fig. 1. Overview of root growth rate experiments. Seeds from each of the 287 double-mutant lines are planted on 1% agar plates, as well as each of the corresponding single-mutants and wild-type. Root growth of seedlings between day four and day six was measured in order to calculate root growth rate. The criteria for identifying significant differences in root growth rate was an unpaired t-test with a significance threshold of $P<0.001$. Double-mutants that showed a significant difference from both corresponding single-mutants were considered as candidates for genetic interaction. The second criteria that we used to screen our quantitative data was the presence of non-overlapping error bars on graphs of the mean growth rates of each double-mutant line and its corresponding single-mutants and wild-type. 26 double-mutants were selected based on these first two criteria. A second, independent root growth rate experiment was performed on these 26 candidates, and the unpaired t-test ($P<0.001$) was used to identify double-mutants with reproducible evidence of genetic interaction.

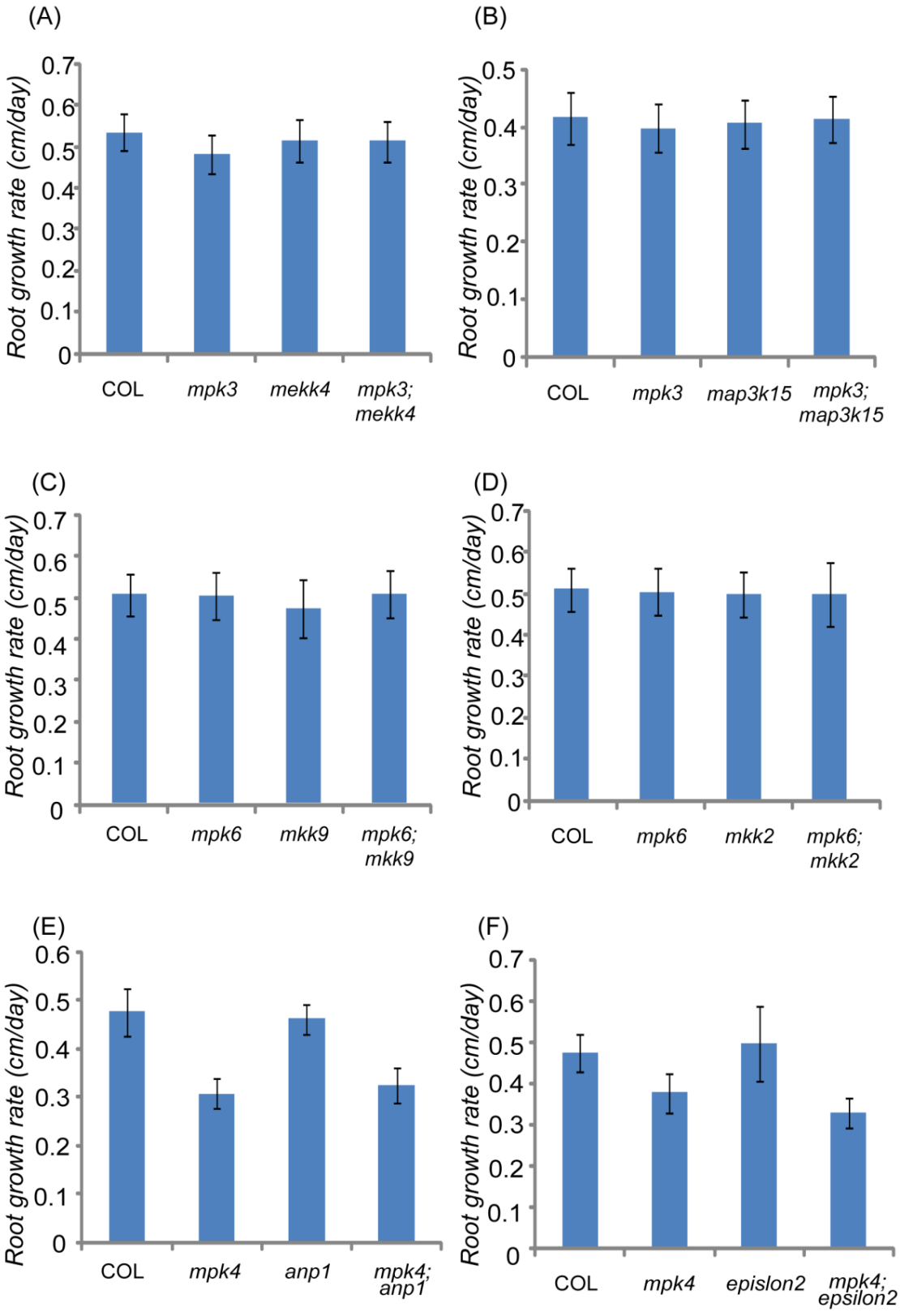

(A)
Root growth rate (cm/day)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
COL
mpk3
mekk4
mpk3; mekk4
(B)
Root growth rate (cm/day)
0.5
0.4
0.3
0.2
0.1
0
COL
mpk3
map3k15
mpk3; map3k15
(C)
Root growth rate (cm/day)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
COL
mpk6
mkk9
mpk6; mkk9
(D)
Root growth rate (cm/day)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
COL
mpk6
mkk2
mpk6; mkk2
(E)
Root growth rate (cm/day)
0.6
0.5
0.4
0.3
0.2
0.1
0
COL
mpk4
anp1
mpk4; anp1
(F)
Root growth rate (cm/day)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
COL
mpk4
epislon2
mpk4; epsilon2

Fig. 2. Representative root growth rate data for double-mutants that do not show evidence of genetic interaction. Each graph shows the rate of root growth per day for each of four genotypes: wild-type, two single-mutants, and a double-mutant. (A,B) Double-mutants involving the *mpk3* query mutant. (C,D) Double-mutants involving the *mpk6* query mutant. (E,F) Double-mutants involving the *mpk4* query mutant. None of the double-mutant lines showed a significant difference in growth rate when compared to the corresponding query mutant (unpaired t-test, $P<0.001$). “COL” indicates wild-type Columbia (Col-0).

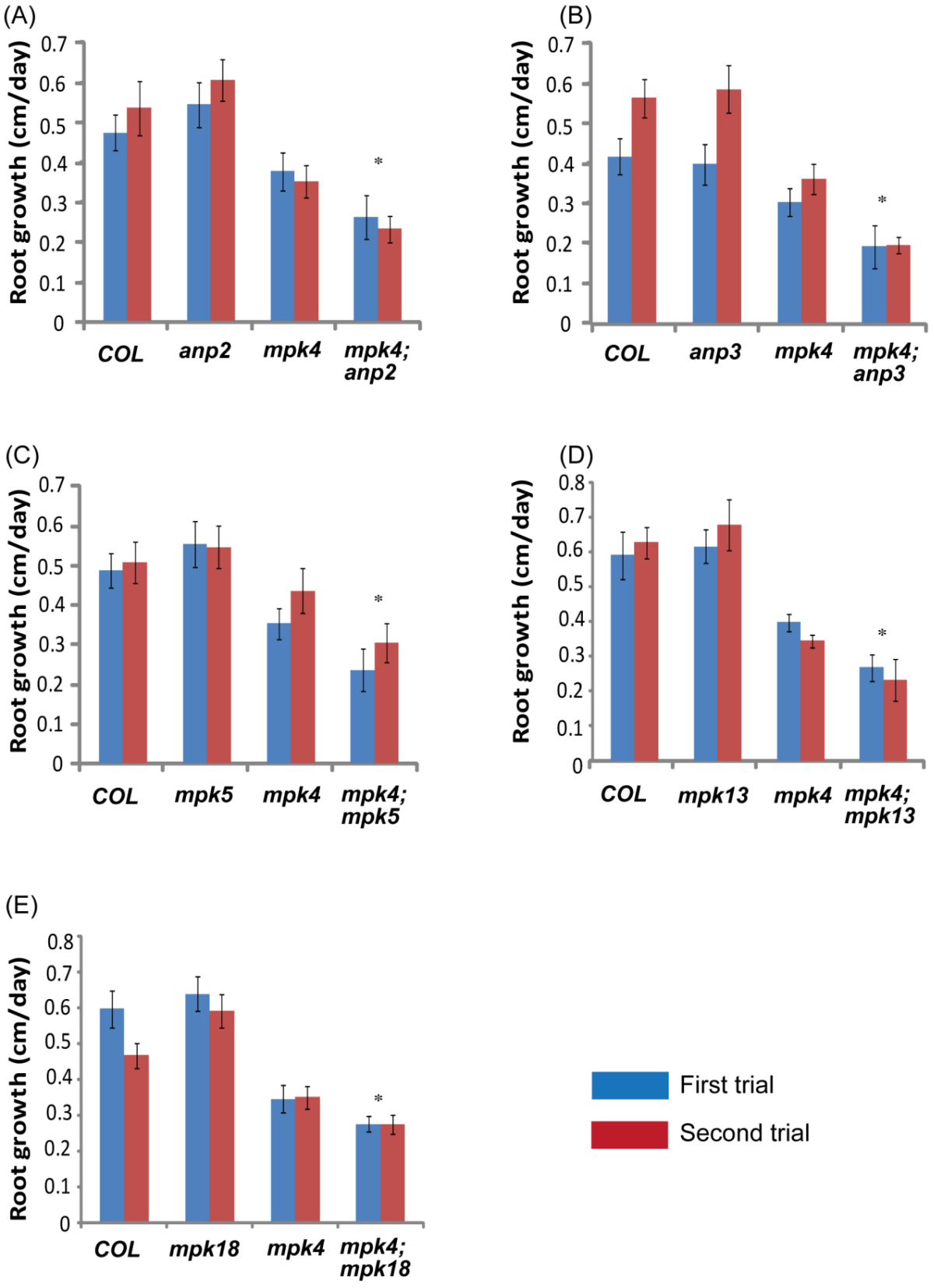

(A)
Root growth (cm/day)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
COL
anp2
mpk4
mpk4; anp2
(B)
Root growth (cm/day)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
COL
anp3
mpk4
mpk4; anp3
(C)
Root growth (cm/day)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
COL
mpk5
mpk4
mpk4; mpk5
(D)
Root growth (cm/day)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
COL
mpk13
mpk4
mpk4; mpk13
(E)
Root growth (cm/day)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
COL
mpk18
mpk4
mpk4; mpk18
First trial
Second trial

Fig. 3. Root growth rate data for double-mutants that show evidence of genetic interaction. Each graph shows the rate of root growth per day in each of four genotypes: wild-type, two single-mutants, and a double-mutant. Blue bars indicate results from the first growth rate experiment, and the red bars indicate results from the second. (A) *ANP2* and *MPK4*. (B) *ANP3* and *MPK4*. (C) *MPK4* and *MPK5*. (D) *MPK4* and *MPK13*. (E) *MPK4* and *MPK18*. All of the double-mutants shown displayed root growth rates that were significantly different (*) from the *mpk4* query mutant (unpaired t-test, $P<0.001$).

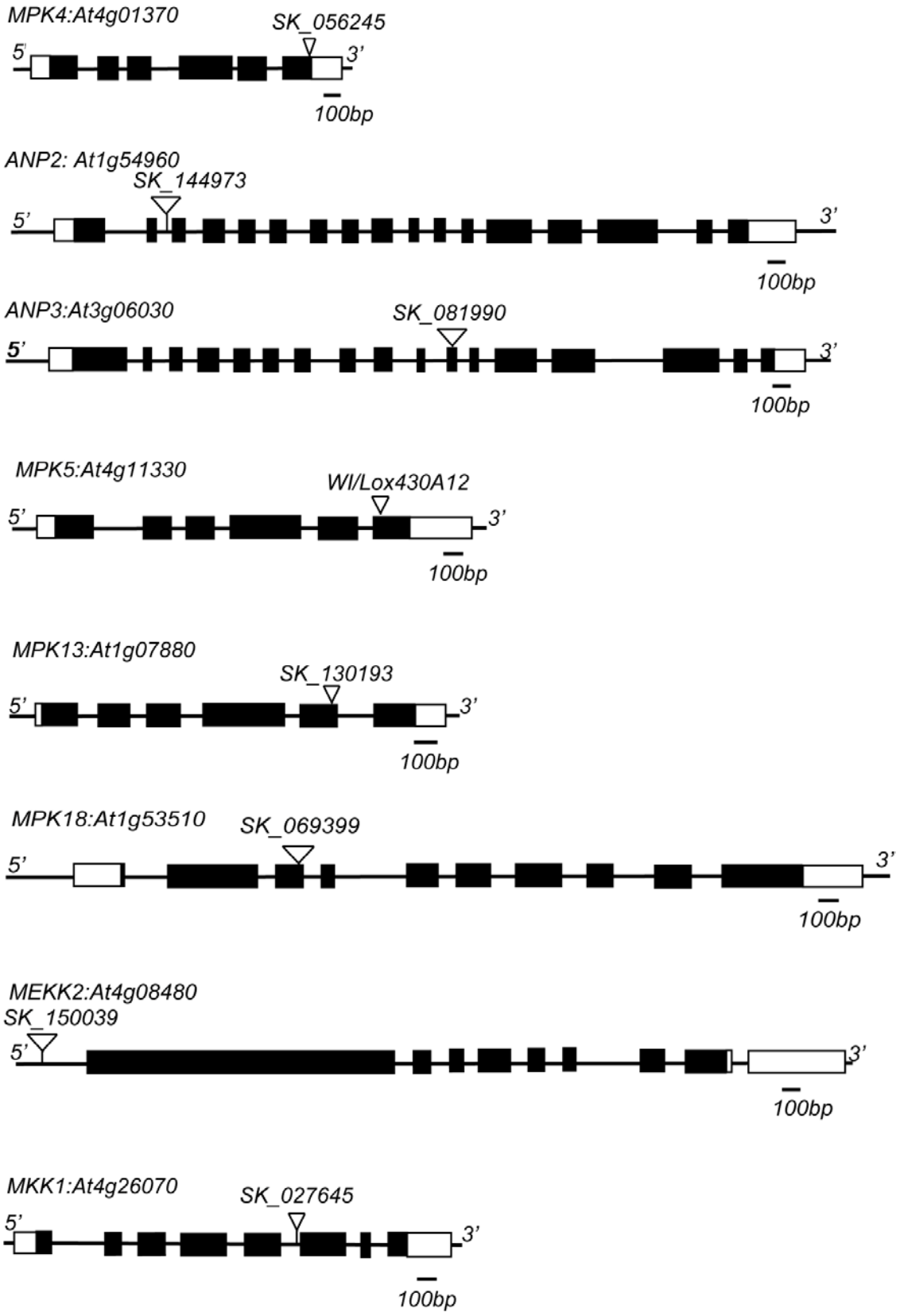
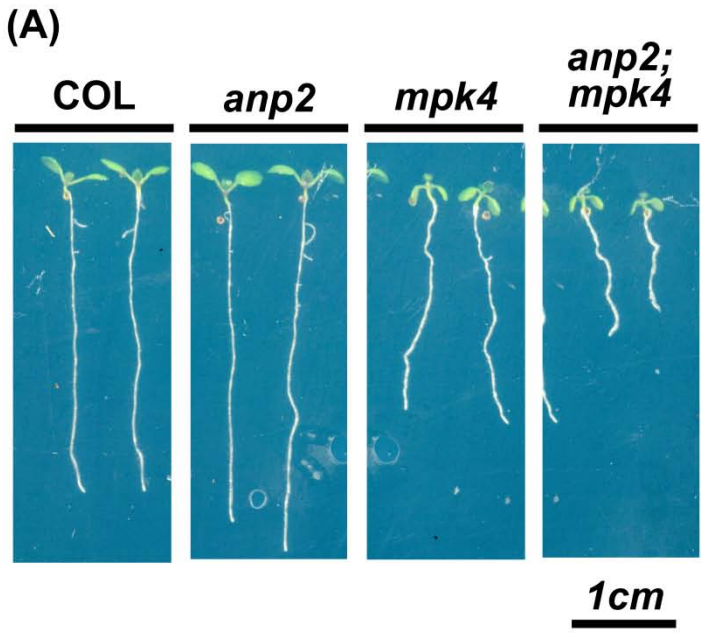

MPK4:At4g01370
SK_056245
5'
3'
100bp
ANP2: At1g54960
SK_144973
5'
3'
100bp
ANP3:At3g06030
SK_081990
5'
3'
100bp
MPK5:At4g11330
WI/Lox430A12
5'
3'
100bp
MPK13:At1g07880
SK_130193
5'
3'
100bp
MPK18:At1g53510
SK_069399
5'
3'
100bp
MEKK2:At4g08480
SK_150039
5'
3'
100bp
MKK1:At4g26070
SK_027645
5'
3'
100bp

Fig. 4. Locations of the T-DNA insertions in the mutant alleles that were used in the genetic interaction screen. Black boxes indicate exons and white boxes indicate transcribed, untranslated regions. Triangles indicate the T-DNA insertion sites. Scale bars are equal to 100bp.


(A)
COL
anp2
mpk4
anp2;
mpk4
1cm

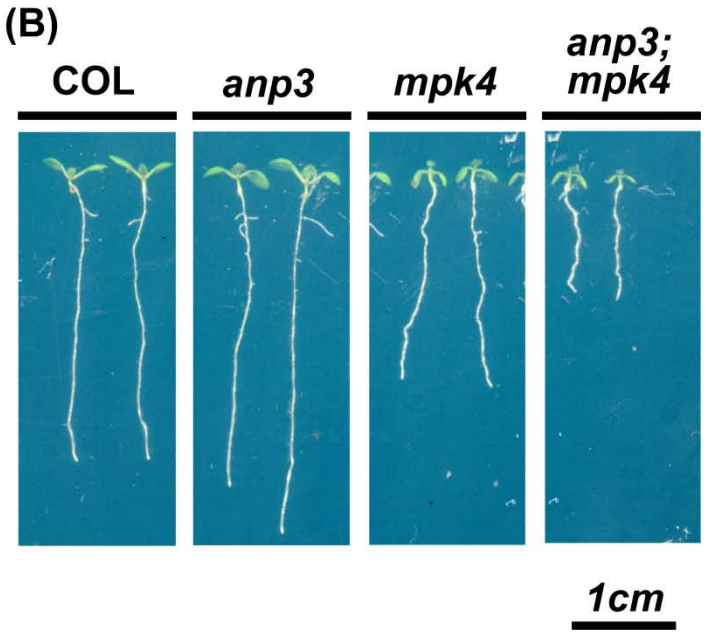

(B)
COL
anp3
mpk4
anp3;
mpk4
1cm

Fig. 5. Representative 6-day-old seedlings grown on vertically oriented agar plates. All mutant loci are homozygous. Scale bars are equal to 1 cm. Seedlings were transferred to a new agar plate for imaging. “COL” indicates wild-type Columbia (Col-0).

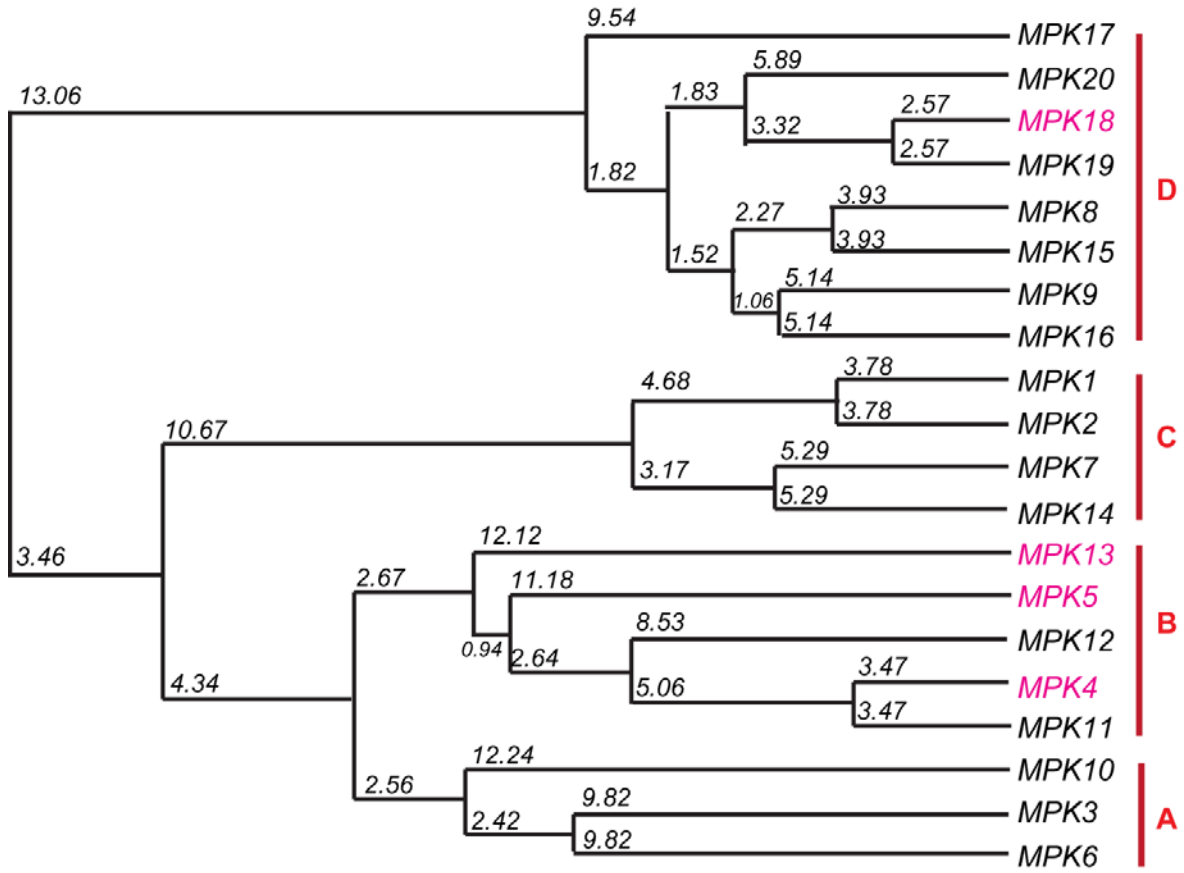

9.54
MPK17
5.89
MPK20
13.06
1.83
2.57
MPK18
3.32
2.57
MPK19
1.82
D
3.93
MPK8
2.27
3.93
MPK15
1.52
5.14
MPK9
1.06
5.14
MPK16
3.78
MPK1
4.68
3.78
MPK2
10.67
5.29
MPK7
3.17
5.29
MPK14
C
12.12
MPK13
3.46
2.67
11.18
MPK5
8.53
MPK12
0.94
2.64
3.47
MPK4
4.34
5.06
3.47
MPK11
B
12.24
MPK10
2.56
9.82
MPK3
2.42
9.82
MPK6
A

Fig. 6. The MAP kinase gene family (Adapted from Bush, 2011). An average distance tree of MAP kinases in Arabidopsis thaliana based on the entire amino acid sequence of each protein was produced using the G-blocks software package. Numbers indicate the percent difference in amino acid identity between proteins. Based on this phylogenetic tree, the 20 MPKs are divided into four sub groups A, B, C, and D (MAPK Group, 2002). Genes highlighted in pink are those for which genetic interactions were identified in this study.

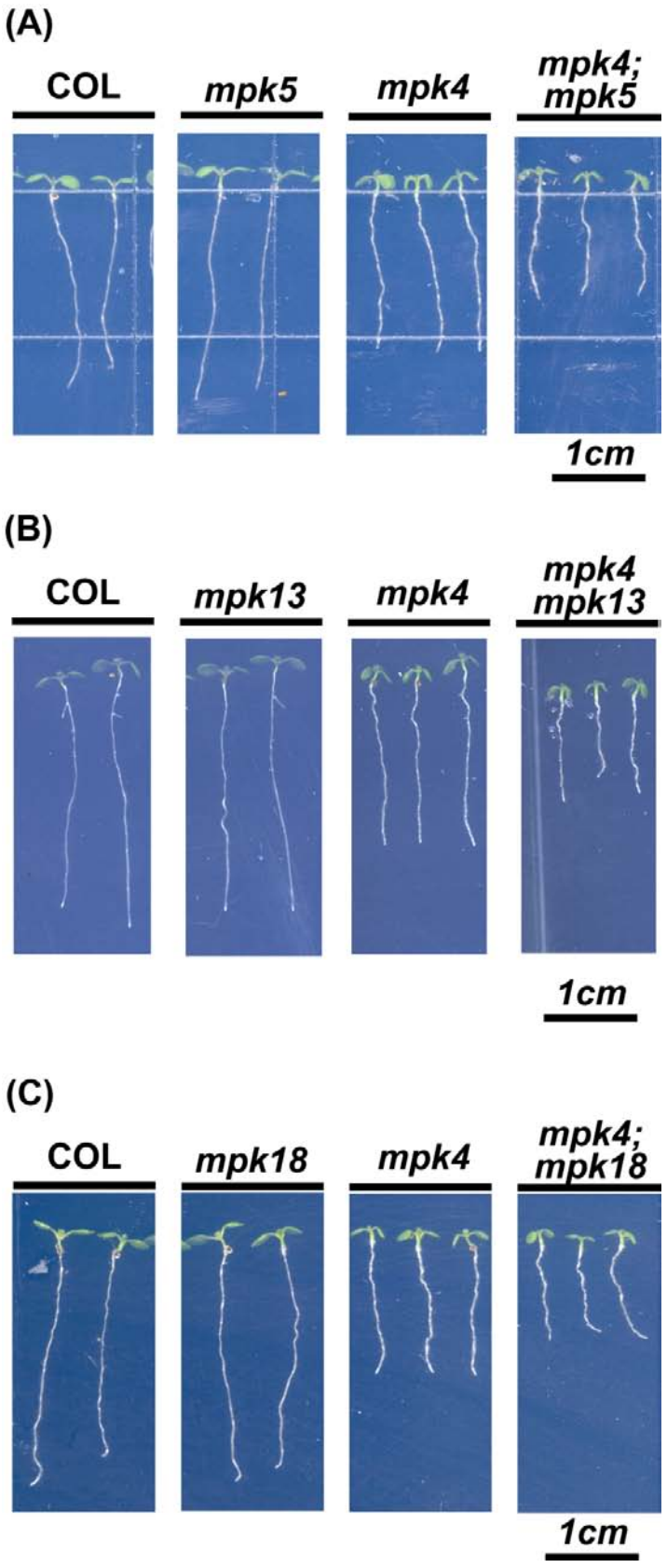

(A)
COL
mpk5
mpk4
mpk4;
mpk5
1cm
(B)
COL
mpk13
mpk4
mpk4
mpk13
1cm
(C)
COL
mpk18
mpk4
mpk4;
mpk18
1cm

Fig. 7. Representative 6-day-old seedlings grown on vertically oriented agar plates. All mutant loci are homozygous. Scale bars are equal to 1 cm. Seedlings were transferred to a new agar plate for imaging. “COL” indicates wild-type Columbia (Col-0).

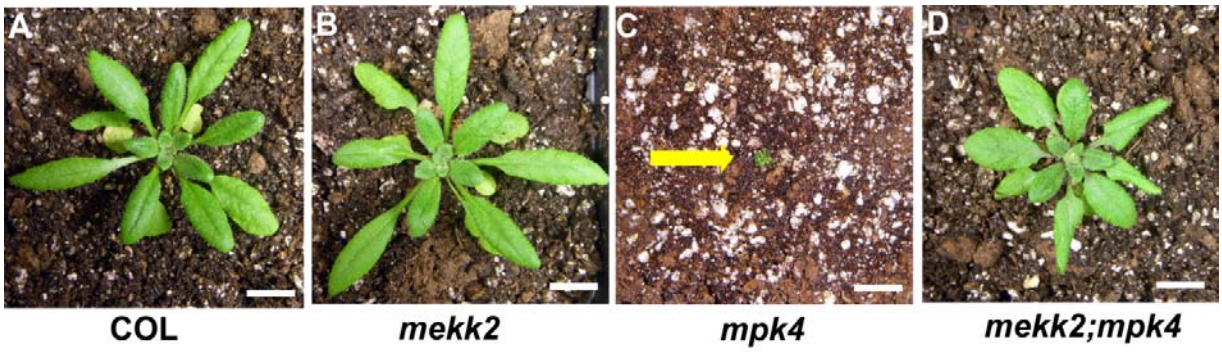

A
B
C
D
COL
mekk2
mpk4
mekk2;mpk4

Fig. 8. The mekk2 mutation rescues the dwarf phenotype of mpk4 single-mutant plants. Representative 21-day-old plants are shown. All mutant loci are homozygous. “COL” indicates wild-type Columbia (Col-0). All images are at the same scale. Scale bars equal 1 cm.

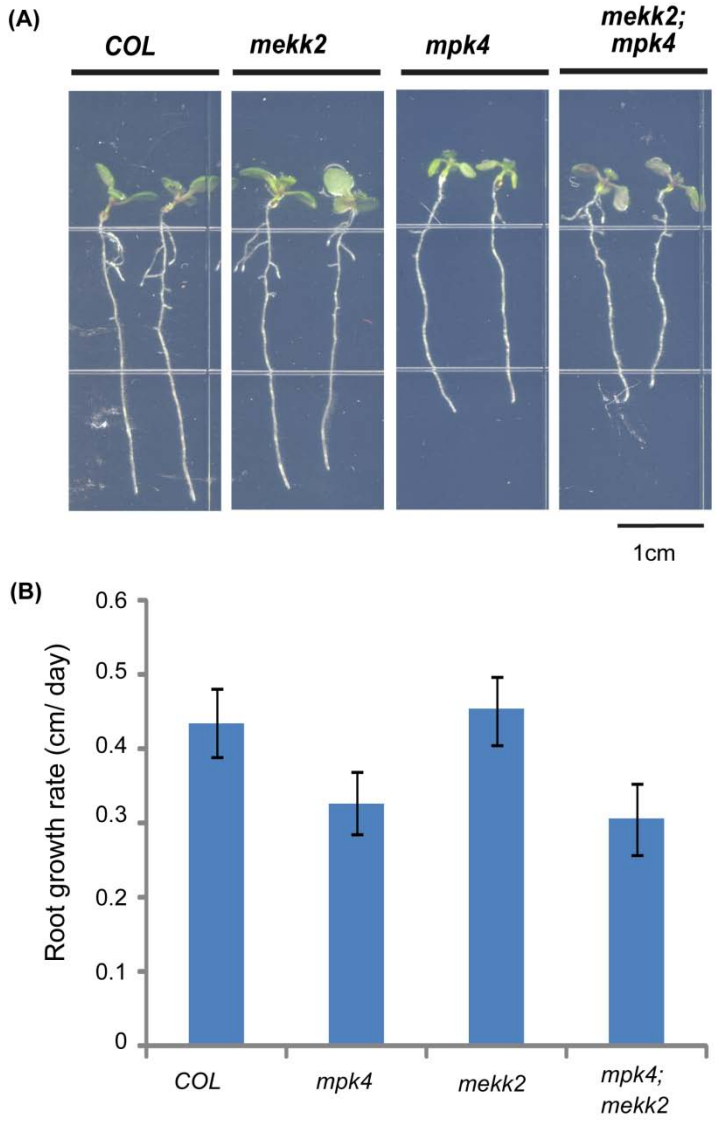

(A)
COL
mekk2
mpk4
mekk2; mpk4
1cm
(B)
Root growth rate (cm/ day)
0.6
0.5
0.4
0.3
0.2
0.1
0
COL
mpk4
mekk2
mpk4; mekk2

Fig. 9. Root growth rates of mekk2;mpk4 double-mutant plants. (A) Seedlings were grown on vertically oriented agar plates. All mutant loci are homozygous. Scale bars are equal to 1 cm. Seedlings were transferred to a new agar plate for imaging. “COL” indicates wild-type Columbia (Col-0). (B) The graph shows the rate of root growth per day for each of the indicated genotypes. The root growth rate of the *mekk2;mpk4* double-mutant was not significantly different from the *mpk4* single-mutant (unpaired t-test, $P<0.001$).

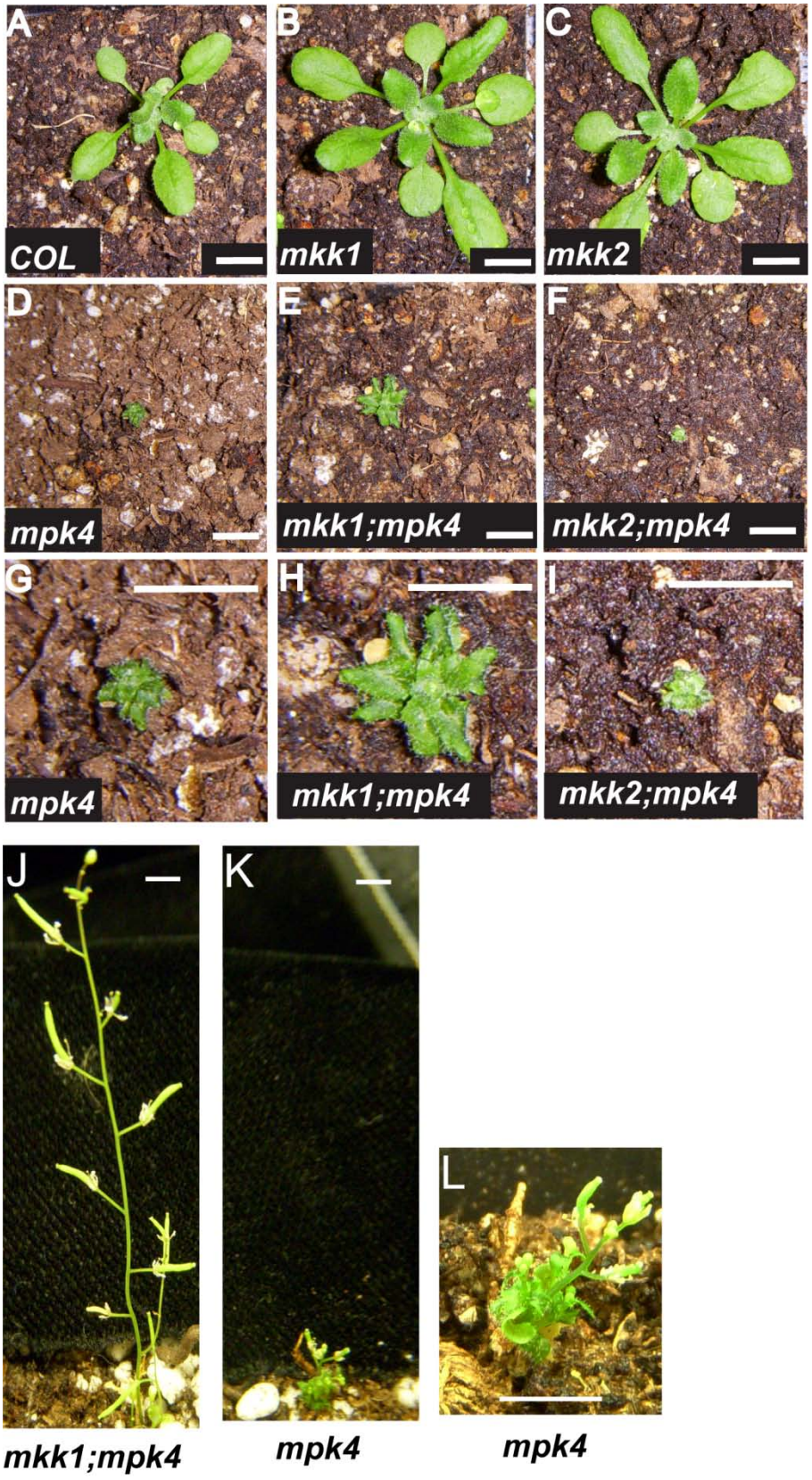

A
COL
B
mkk1
C
mkk2
D
mpk4
E
mkk1;mpk4
F
mkk2;mpk4
G
mpk4
H
mkk1;mpk4
I
mkk2;mpk4
J
mkk1;mpk4
K
mpk4
L
mpk4

Fig. 10. The mkk1 mutation partially rescues the dwarf phenotype of mpk4 mutant plants. (A-C) 18-day-old plants of wild-type Columbia (COL), *mkk1* single-mutant, and *mkk2* single-mutant; (D-F) 18 day-old plants of *mpk4, mkk1;mpk4* double-mutant, and *mkk2;mpk4* double-mutant. Images A-F are shown at the same scale. (G-I) Higher magnification images of the plants shown in D-F. (J) Representative 45-day-old mkk1;mpk4 double-homozygous mutant plants. (K) Representative 45-day-old mpk4 single-mutant plants. (L) Higher magnification image of the 45-day-old mpk4 single-mutant plant shown in (K). All scale bars equal 1 cm. All mutant loci are homozygous.

Table 1. List of the candidates of genetic interaction in screening

Query Gene	Target Gene	Double-mutant ID	Phenotype of double-mutants
MPK3	*MPK6*	SH1015a	Embryo lethal (Wang et al., 2007)
MPK4	*MEKK1*	SH0001a	Seedling lethal (Su et al., 2007)
MPK4	*MEKK2*	SH0355b	Dwarf phenotype rescued
MPK4	*ANP2*	SH0233b	Root growth reduction;
MPK4	*ANP3*	SH0005b	Root growth reduction;
MPK4	*MKK1*	SH0268b	Dwarf phenotype partially rescued
MPK4	*MPK5*	SH1024b	Root growth reduction
MPK4	*MPK13*	SH1028b	Root growth reduction
MPK4	*MPK18*	SH1032b	Root growth reduction

"Double-mutant ID" indicates the seed stock number used for this project. For double-mutants that contain *mpk4*, the phenotype reported above is defined relative to the *mpk4* single mutant.

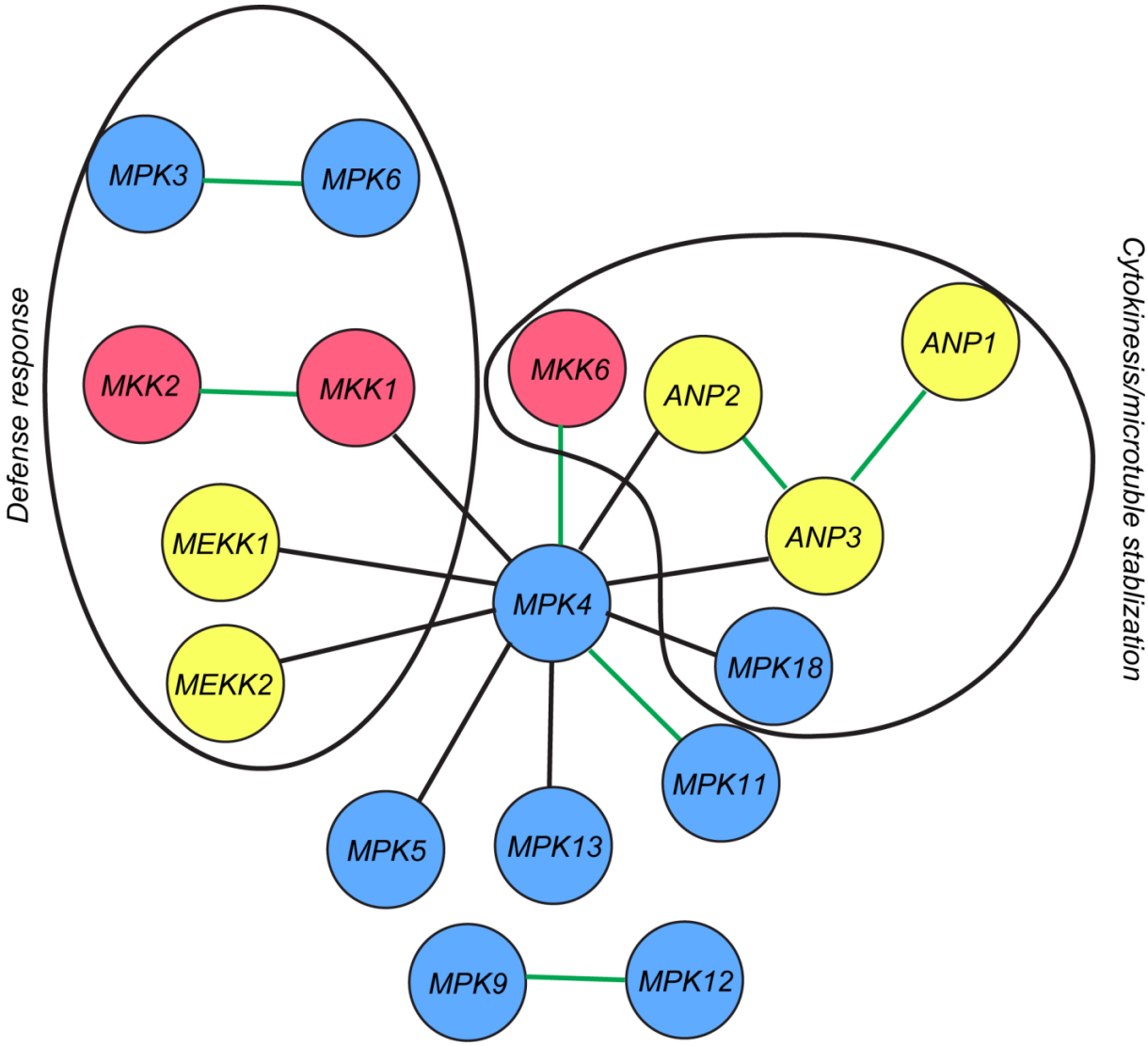

Defense response
Cytokinesis/microtuble stablization
MPK3
MPK6
MKK2
MKK1
MEKK1
MEKK2
MPK4
MKK6
ANP2
ANP1
ANP3
MPK18
MPK11
MPK5
MPK13
MPK9
MPK12

Fig. 11. Genetic interaction network of MAPK-related genes in Arabidopsis. Each node represents a gene and each edge refers to a genetic interaction. Blue circles represent *MAP kinases*; pink circles represent *MAP2Ks*, and yellow circles represent *MAP3Ks.* Black lines indicate genetic interactions identified in this project. Green lines indicate interactions previously reported in the literature.

LITERATURE CITED

Bush, M. (2010) Functional genomic analysis of mitogen-activated protein kinases in Arabiodpsis thaliana. PhD thesis. University of Wisconsin, Madison, Wisconsin.

Byrne, A.B., Weirauch, M.T., Wong, V., Koeva, M., Dixon, S.J., Stuart, J.M., and Roy, P.J. (2007) A global analysis of genetic interactions in Caenorhabditis elegans. J Biol. 6:8.

Clark, K.A., and Krysan, P.J. (2010) Chromosomal translocations are a common phenomenon in Arabidopsis thaliana T-DNA insertion lines. Plant J. 2010 64:990-1001

Dixon, S.J., Costanzo, M., Baryshnikova, A., Andrews, B., and Boone, C. (2009) Systematic mapping of genetic interaction networks. Annu. Rev. Genet. 43:601-625.

Ichimura, K., Casais, C., Peck, S.C., Shinozaki, K. and Shirasu, K. (2006) MEKK1 is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. J. Biol. Chem. 281, 36969–36976.

Kosetsu, K.K., Matsunage, S., Nakagami, H., Colcombet, J., Sasabe, M., Soyano, T., Takahashi, Y., and Machida, Y. (2010) The MAP kinase MPK4 is required for Cytokinesis in Arabidopsis thaliana. Plant Cell 22: 3778-3790.

Krysan, P.J., Jester, P.J., Gottwald, J.R., and Sussman, M.R. (2002) An Arabidopsis mitogen- activated protein kinase kinase kinase gene family encodes essential positive regulators of cytokinesis. Plant Cell 14:1109-1120.

Lehner, B., Crombie, C., Tischler, J., Fortunato, A., and Fraser, A. G. (2006) Systematic mapping of genetic interactions in Caenorhabditis elegans identifies common modifiers of diverse signaling pathways. Nature Genetics 38: 896 - 903

MAPK Group. 2002. Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends in Plant Science, 7: 301-308.

Melikant, B., Giuliani, C., Halbmayer-Watzina, S., Limmongkon, A., Heberle-Bors, E., Wilson, C. (2004) The Arabidopsis thaliana MEK AtMKK6 activates the MAP kinase AtMPK13. FEBS Letters 576: 5–8

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J. (2000) Arabidopsis MAP Kinase 4 negatively regulates systemic acquired resistance. Cell 103: 1111-1120.

Qiu, J.L., Zhou, L., Yun, B.W., Nielsen, H.B., Fiil, B.K., Petersen, K., Mackinlay, J., Loake, G.J., Mundy, J., Morris, P.C. (2008b) Arabidopsis mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. Plant Physiol 148: 212-222.

Rasband, W.S., (1997-2011) ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/

Su, S-H., Suarez-Rodriguez, M.C., and Krysan, P. (2007) Genetic interaction and phenotypic analysis of the Arabidopsis MAP kinase pathway mutations mekk1 and mpk4 suggests signaling pathway complexity. FEBS Letters 581: 3171-3177.

Suarez-Rodriguez, M.C., Adams-Phillips, L., Liu, Y., Wang, H., Su, S.H., Jester, P.J., Zhang, S., Bent, A.F., Krysan, P.J. (2007) MEKK1 is required for flg22-induced MPK4 activation in Arabidopsis plants. Plant Physiol. 143, 661–669.

Teige, M., Scheikl, E., Eulgem, T., Dóczi, R., Ichimura, K., Shinozaki, K., Dangl, J.L., Hirt, H. (2004) The MKK2 Pathway Mediates Cold and Salt Stress Signaling in Arabidopsis. Molecular Cell 15: 141-152.

Walia, Ankit.,. Lee, J. S., Wasteneys, G., Ellis, B. (2009) Arabidopsis mitogen-activated protein kinase MPK18 mediates cortical microtubule functions in plant cells. Plant J. 59:565-575.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C., Zhang, S. (2007) Stomatal Development and Patterning Are Regulated by Environmentally Responsive Mitogen-Activated Protein Kinases in Arabidopsis. Plant Cell 19: 63-73.

Xing, Y., Jia, W., and Zhang, J. (2009) AtMKK1 and AtMPK6 are involved in abscisic acid and sugar signaling inArabidopsis seed germination. Plant Mol. Bio. 70:725-736.