Abstract

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Title: A genetic approach to understanding co-regulated MAPK genes in Arabidopsis thaliana

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COVER SHEET

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A genetic approach to understanding co-regulated MAPK genes in *Arabidopsis thaliana*

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Abstract

Mitogen-activated protein kinase (MAPK) signaling cascades connect cellular signal and gene transcription in cells. The pathway can regulate cell division, cell growth, and other pathways in eukaryotes. We used the Comprehensive Systems Biology Project database to find out that *MPK1, 2,* and *6* have a similar co-response level, as do *MPK8, 19* and *20*. We hypothesized that the genes in each group serve similar functions and would respond to stress similarly in *Arabidopsis*. We created a homozygous knockout mutant in *MPK1, 2* and *6*, and another one in *MPK8, 19* and *20*. Under normal growth conditions, the triple mutant seedlings appeared identical to wildtype seedlings. We compared the mutant and wildtype seedlings in different stress conditions (0.3 uM kinetin, 2% sucrose, 5% sorbitol, ethylene and 14°C chilling). We measured root growth of light-grown seedlings in the first three and chill treatments and hypocotyl length for sucrose-treated and ethylene-treated dark-grown seedlings. Results showed that there was no significant difference in the root or hypocotyl length growth between wildtype and mutant seedlings in all the experimental conditions. Thus, *MPK1, 2,* and 6 are not required in responding to stress based on the *MPK1, 2, 6* triple mutant; *MPK8, 19 and 20* are not required in the stress response cased on the *MPK8, 19, 20* triple mutant. Future experiments have to be done to give information of their functions. This project shows a reverse genetic approach to study gene function.

Introduction

Mitogen-activated protein kinases (MAPKs) are a type of kinase which is able to transfer a phosphate group from ATP to the target molecule for activation. The MAPK signaling cascade is a series of phosphorylation events that serve as the bridge linking cellular receptors to the control of cell proliferation, cell growth, differentiation, defense mechanism and stress response in eukaryotes. When the receptor on the cell surface is activated by an extracellular ligand, it activates MAP kinase kinase kinase (MAPKKK or MEKK). MAPKKK then phosphorylates and activates MAPKK which can activate MAPK. The downstream end of the pathway is a number of transcription factors which control gene expression in the nucleus (Fig.1). The bacterial flagellin pathway which activates the pathogen resistance in *Arabidopsis* is an example of the MAPK pathway (Jonak *et.al* 2002). In eukaryotes, the three main categories of MAPKs are stress-activated MAPK (JNK or SAPK), extracellular signal-activated MAPK (ERK) and p38, which is activated by endotoxin (Kultz 1998). All MAPKs in plants belong to the ERK family and are able to respond to signals other than mitogens. On the other hand, mammalian ERKs are only able to respond to mitogens (Tena *et.al* 2001).

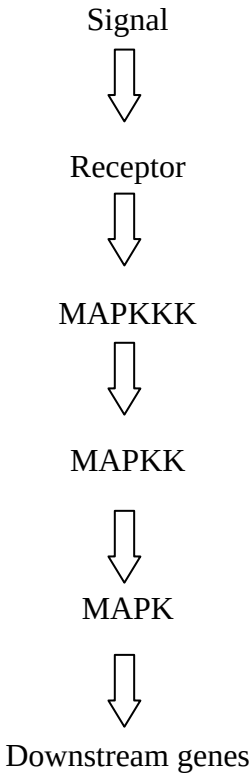

Figure 1. MAPK signaling cascade. Cellular signal activates the receptor and triggers the MAPK pathway. MAP kinase kinase kinase activates MAP kinase kinase by phosphorylation. MAPKK then activates MAPK and the regulation of the downstream genes.

The MAPK signaling cascades in *Arabidopsis thaliana* are the pathways that Krysan's lab is studying. There are a total of 20 MAPK genes (*MPK*) and are classified into four main classes based on their phylogenetic relationship and the similarities in the amino acid sequences, as shown in the phylogenetic tree of Arabidopsis *MPK* genes (Fig.2). The bolded *MPK* genes in the tree have been characterized genetically recently. The six *MPK* genes highlighted by the red asterisks were the focus of my project.

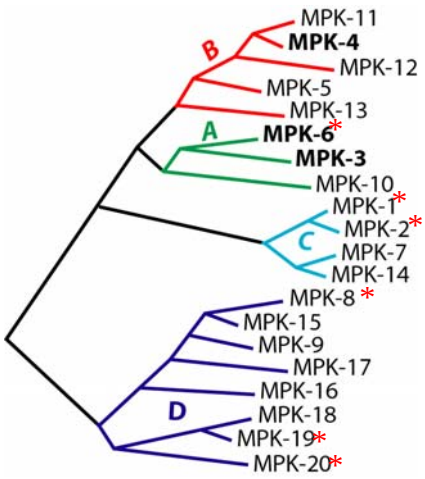

Figure 2: The Arabidopsis MPK phylogenetic tree. The tree shows the classification of the 20 *MPK* genes in *Arabidopsis thaliana* based on the similarities of amino acid sequences (MAPK group 2002). The six *MPK* genes highlighted by red asterisks were the focus in the project.

Instead of following the phylogenetic relationship of the 20 *MPK* genes in Figure 2, my study took a reverse genetic approach in studying the *MPK* functions. My project started with the Comprehensive Systems Biology (CSB) Project done by the Max Planck Institute of Molecular Plant Physiology. This online database provides the data of transcript co-response analysis in *Arabidopsis thaliana*. Researchers planted wildtype *Arabidopsis* under different developmental and stress conditions and extracted the RNA from plants for performing the expression profiling in microarray. They provided the transcriptional levels of different genes in certain parts of the plants under a certain environment and calculated the Spearman coefficient as an indicator of how much each gene pair correlated with each other. The calculation of Spearman coefficient takes into account the difference in mRNA expression values of each gene pair under a specific environment and the number of experimental conditions. A higher Spearman coefficient indicates the gene pair has highly similar gene expression patterns. After inputting the 20 *Arabidopsis MPK* genes into the database, *MPK1* and *2* had the highest Spearman coefficient of 0.5568; *MPK8* and *19* had the coefficient of 0.5431; *MPK2* and *6* had 0.5112; *MPK8* and *20* had 0.0044 (Fig.3). Based on the available mutant alleles and the high coefficient gene pairs, I assumed that *MPK1, 2,* and *6* are co-regulated and so do *MPK8, 19* and *20*. The *MPK* mutants in my project have a T-DNA insert which is much larger than the size of the *MPK* genes. As the large size of T-DNA cannot be transcribed by the ribosome polymerase and the transcription stops before reaching the stop codon in the genes, the gene cannot be transcribed and the function is lost. All *MPK* mutants were shown to be null allelles by using RT-PCR (Clark and Bush 2006). Thus, I decided to create a plant that was homozygous knockout mutant in *MPK1, 2* and *6* and another plant that was homozygous knockout mutant in *MPK8, 19* and *20.* Then I would compare their phenotypes with wildtype *Arabidopsis* under several experimental conditions. By comparing with wildtype plants, any discrepancies on the phenotypes would provide information on the functions

of the mutant *MPK* genes. Based on the high correlation among *MPK1, 2, and 6*, also among *MPK8, 19* and *20*, my hypothesis was that the genes in each group serve similar functions in *Arabidopsis* and would respond to various stress environments in a similar way.

Gene pair	Spearman Coefficient
MPK1, MPK2	0.5568
MPK8, MPK19	0.5431
MPK2, MPK6	0.5112
MPK8, MPK20	0.0044

Figure 3. Table showing the Spearman coefficients of MPK genes involved.

The experimental conditions done in my project can be referred to the 2010 Gauntlet Project from the Western Washington University. Researchers in the project plated wildtype *Arabidopsis* seeds in different stress conditions, including hormonal addition, nutrient addition, environmental stress and developmental observations of the seedlings. They measured the root or hypocotyl lengths of seedlings in order to determine how wildtype *Arabidopsis* would respond to stress. Detailed procedures and the results of measurements are available on their website. I picked five experimental conditions that were fairly plausible in terms of time and available resources in the lab. They were 0.3uM kinetin, 2% (w/v) sucrose, 5% (w/v) sorbitol, ethylene (25uM ACC) and 14° C chilling.

Methods and Materials

For creating the *MPK1, 2, 6* triple mutant, I made crosses between *MPK1* and *MPK2* single mutants, also between *MPK2* and *MPK6* single mutants (Fig.4). The resulting double heterozygous from the two crosses (*mpk1 / MPK1; mpk2 / MPK2* and *mpk2 / MPK2 ; mpk6 / MPK6*) were crossed to produce a plant that was heterozygous at *MPK1* and *6* and homozygous mutant at *MPK2*. The self-cross of this plant created the *MPK1, 2, 6* triple mutant (*mpk1 / mpk1; mpk2 / mpk2; mpk6 /mpk6*).

For creating the *MPK8, 19, 20* triple mutant, I first made a self-cross of the double heterozygous of *MPK8* and *20* (*mpk8 / MPK8; mpk20 / MPK20*). The resulting double homozygous mutant of *MPK8* and *20* was then crossed to another homozygous double mutant of *MPK19* and *20* to obtain a plant that was heterozygous at *MPK8* and *19* and mutant at *MPK20* (*mpk8 / MPK8 ; mpk19 / MPK19 ; mpk20 / mpk20*). After self-crossing this plant, the *MPK8, 19, 20* triple mutant (*mpk8 / mpk8; mpk19 / mpk19; mpk20 / mpk20*) was produced. All mutant plants were identified by PCR. Fluorescent bands were seen in wildtype and heterozygous plants when forward and reverse primers were used because they have chromosomes without the TDNA inserts. The normal primers cannot transcribe the whole huge inserts in the mutants and thus no DNA can be replicated. When forward and TDNA primers were added, only heterozygous and mutant plants had bands in the gel because they have TDNA inserts. By comparing the two gels with different combinations of primers, mutant plants were identified throughout all crosses.

MPK1, 2, 6 triple mutant:

mpk1/ mpk1 X *mpk2 / mpk2* *mpk2 / mpk2* X *mpk6 / mpk6*

↓ ↓

mpk1 / MPK1 ; mpk2 / MPK2 X *mpk2 / MPK2 ; mpk6 / MPK6*

↓

mpk1 / MPK1 ; mpk2 / mpk2 ; mpk6 / MPK6 X *self*

↓

<u>mpk1 / mpk1 ; mpk2 / mpk2 ; mpk6 / mpk6</u>

MPK8, 19, 20 triple mutant:

mpk8 / MPK8 ; mpk20 / MPK20 X *mpk8 / MPK8 ; mpk20 / MPK20*

↓

mpk8 / mpk8 ; mpk20 / mpk20 X

mpk19 / mpk19 ; mpk20 / mpk20

↓

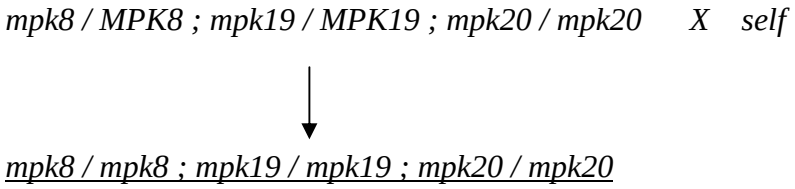

Figure 4. Crossing schemes of creating the MPK1, 2, 6 and MPK8, 19, 20 triple mutants.

Both the *MPK1, 2, 6*, and *MPK8, 19, 20* triple mutant seeds were plated in different conditions and compared with wildtype seeds (*Columbia*) to explore the functions of the *MPK* genes involved in this study. The two triple mutants looked identical to wildtype *Arabidopsis* under normal conditions (grown in 21° C growth room and on MS media plates) The five experimental conditions included the addition of 0.3uM kinetin, 2% (w/v) sucrose, 5% (w/v) sorbitol, ethylene and chilling at 14° C. Concentrations used in the conditions are suggested by the 2010 Gantlet Project for the following observations: 0.3uM of kinetin was found to result in 50% root length reduction in wildtype seeds; 2% (w/v) sucrose could increase hypocotyl growth to 20% in dark and increase root growth slightly in light in wildtype; 5% (w/v) sorbitol reduced root length growth to about 50% in wildtype; ethylene reduced hypocotyl growth to about only one fifth of normal length in the dark. The effects of 14° C chill assay on wildtype plants were not provided.

For my assays, sterilized mutant and wildtype seeds were plated on different sides of each 0.5X MS media plate and experimental plate for comparison. In the ethylene assay, ACC solution was added to the plate and was converted to ethylene gas by the ACC oxidase in the seedlings. After incubating at 4°C wrapped in aluminum foil for 3-5 days, the plates were placed in the 21 ° C growth room. Pictures of plates were taken after 5-7 days of seed growth in the experimental conditions. For the 14° C chill assay, the wildype and mutant seeds were plated on MS media plates only. After incubating in the fridge in foil for 3-5 days, half of the plates were placed in the

14° C incubator and half were placed in the 21° C growth room as control.

I measured the root length growth of seedlings plated in 0.3uM kinetin, 2% (w/v) sucrose, 5% (w/v) sorbitol, ethylene and chilling at 14° C. These plates were placed in the light during growth. I also measured hypocotyl growth for the seedlings grown in sucrose and ethylene. They were wrapped in layers of foil to avoid the light after taken out from the fridge. All the plates were scanned and the lengths of roots or hypocotyls were measured by the Image J program. The seeds measured in the MS media plates were considered as 100 % growth and those grown in experimental conditions were measured as a percentage of the control seedlings.

Results

The *MPK1, 2, 6* and *MPK8, 19, 20* triple mutant seeds showed same phenotype as wildtype seedlings under normal growth conditions (grown on 0.5X MS media plates at 21° C). Root length reduction of around 40-50% was observed in wildtype and the two mutant lines (Fig.5). There was no significant difference between the wildtype and mutant seedlings in root length.

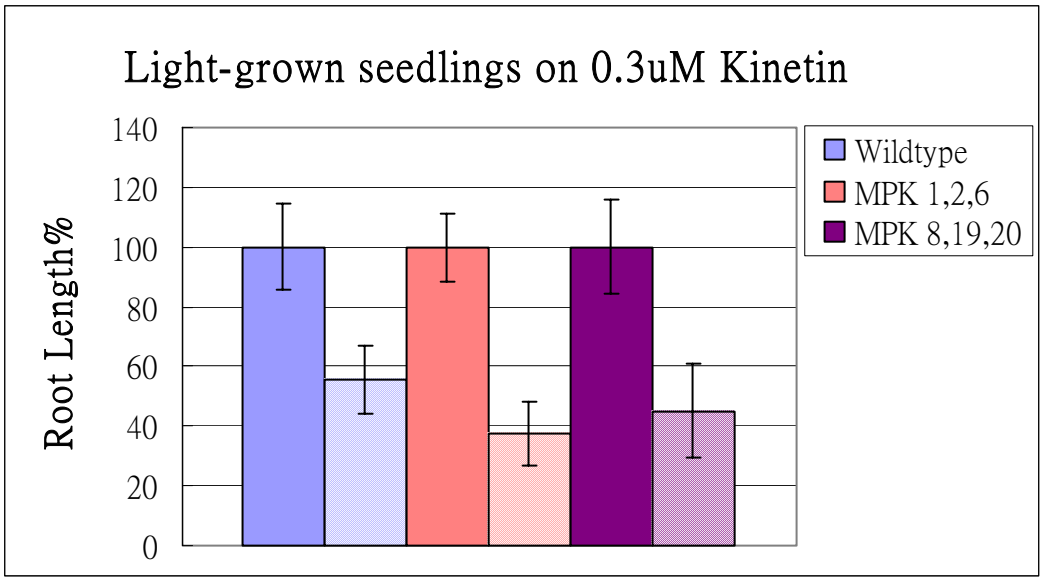

Figure 5. The root growth reduction of wildtype and mutant seeds on 0.3uM kinetin. Wildtype, *MPK1,2,6* and *MPK8,19,20* mutant seeds were plated on 0.3uM and 0.5X MS media plates in the light for 5-7 days. The root lengths of seeds grown on MS media were considered as 100% root growth. The columns with solid colors represent the root growth of plants plated on MS media. The striped bars are the 0.3uM kinetin plates.

The three types of seeds were grown on 2% (w/v) sucrose plates for observation of hypocotyl length in the dark and root length in the light. When grown in the dark, the hypocotyls of all seedlings on sucrose plates showed about 70% of those grown on MS plates (Fig.6). There was no significant difference in hypocotyl growth.

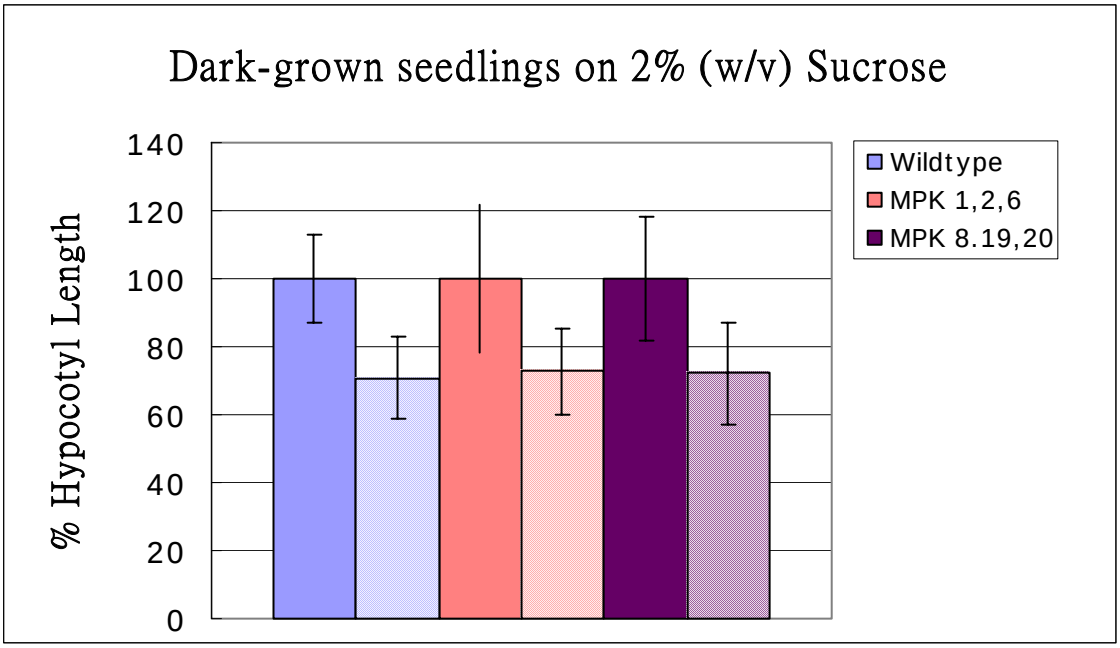

Figure 6. The hypocotyl growth reduction of wildtype and mutant seeds on 2% (w/v) sucrose. Wildtype, *MPK1,2,6* and *MPK8,19,20* mutant seeds were plated on 2% (w/v) sucrose and 0.5X MS media plates in the dark for 5-7 days after being exposed to light for 2 hours. The hypocotyl lengths of seeds grown on MS media were considered as 100% hypocotyl growth. The columns with solid colors represent the root growth of plants plated on MS media. The striped bars are the sucrose plates.

Wildtype and mutant seeds grown on 2% (w/v) sucrose showed almost twice the root growth of the seeds grown on MS media (Fig.7). The mutants showed no significant difference compared to the control seedlings.

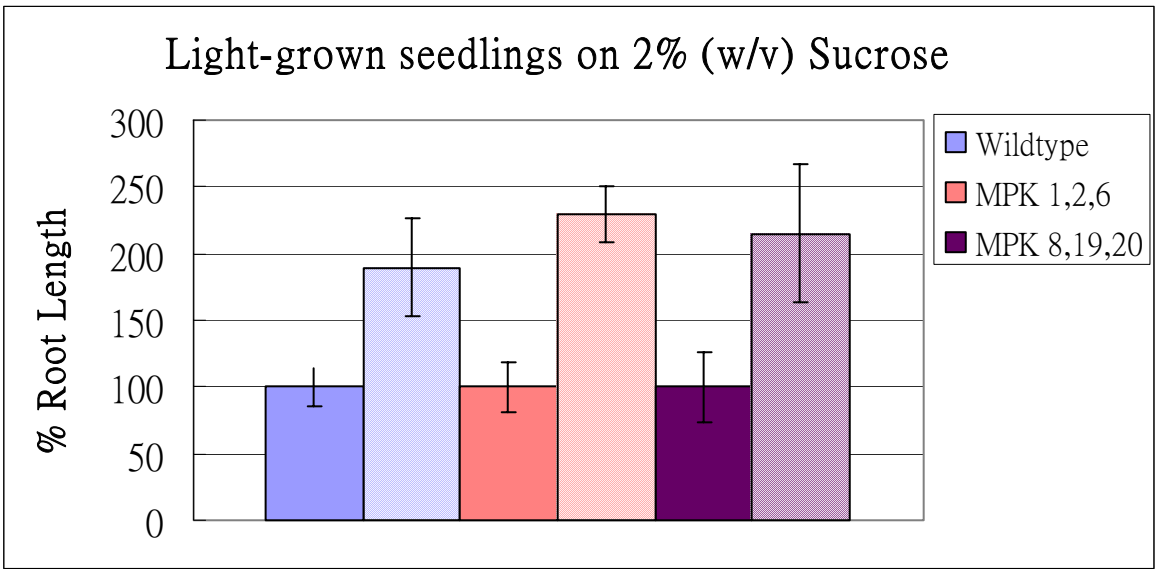

Figure 7. The root growth reduction of wildtype and mutant seeds on 2% (w/v) sucrose. Wildtype, *MPK1,2,6* and *MPK8,19,20* mutant seeds were plated on 2% (w/v) sucrose and 0.5X MS media plates in the light for 5-7 days. The root lengths of seeds grown on MS media were considered as 100% root growth. The columns with solid colors represent the root growth of plants plated on MS media. The striped bars are the sucrose plates.

For the 5% (w/v) sorbitol assay, wildtype and mutant seedlings had only about 30% of the root length compared to those grown on MS media plates (Fig.8). The phenotypes of *MPK1, 2, 6* and *MPK8, 19, 20* mutant seedlings had no significant difference.

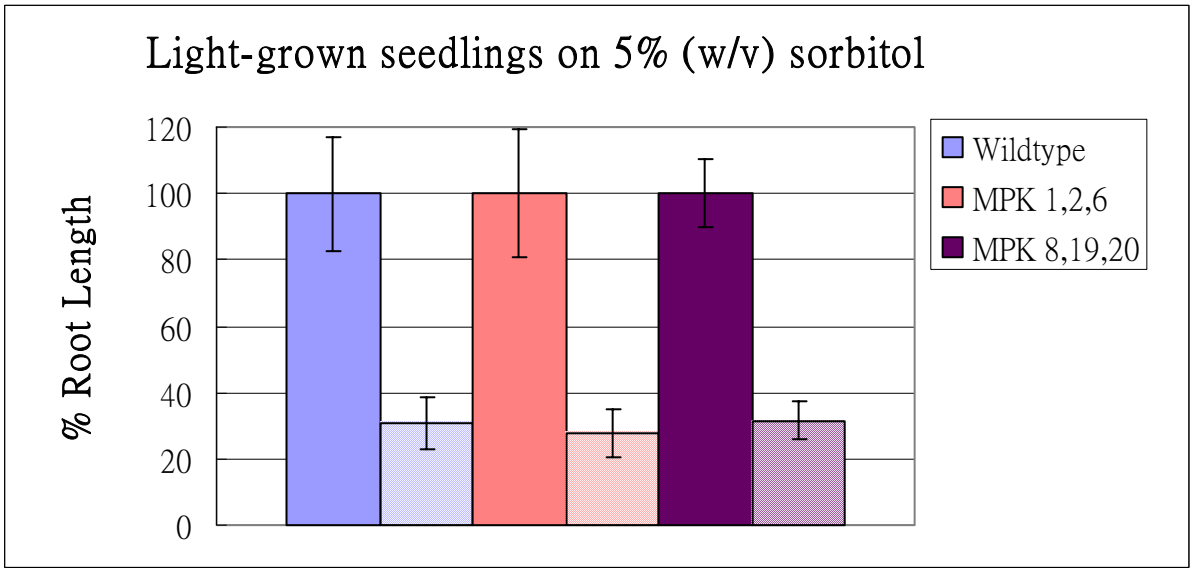

Figure 8. The root growth reduction of wildtype and mutant seeds on 5% (w/v) sorbitol. Wildtype, *MPK1,2,6* and *MPK8,19,20* mutant seeds were plated on 5% (w/v) sorbitol and 0.5X MS media plates in the light for 5-7 days. The root lengths of seeds grown on MS media were considered as 100% root growth. The columns with solid colors represent the root growth of plants plated on MS media. The striped bars are the sorbitol plates.

All three types of seedlings showed about only 40% growth of hypocotyl length when grown

on ethylene (Fig.9). There was no significant difference in hypocotyl growth between the wildtype and the two triple mutants.

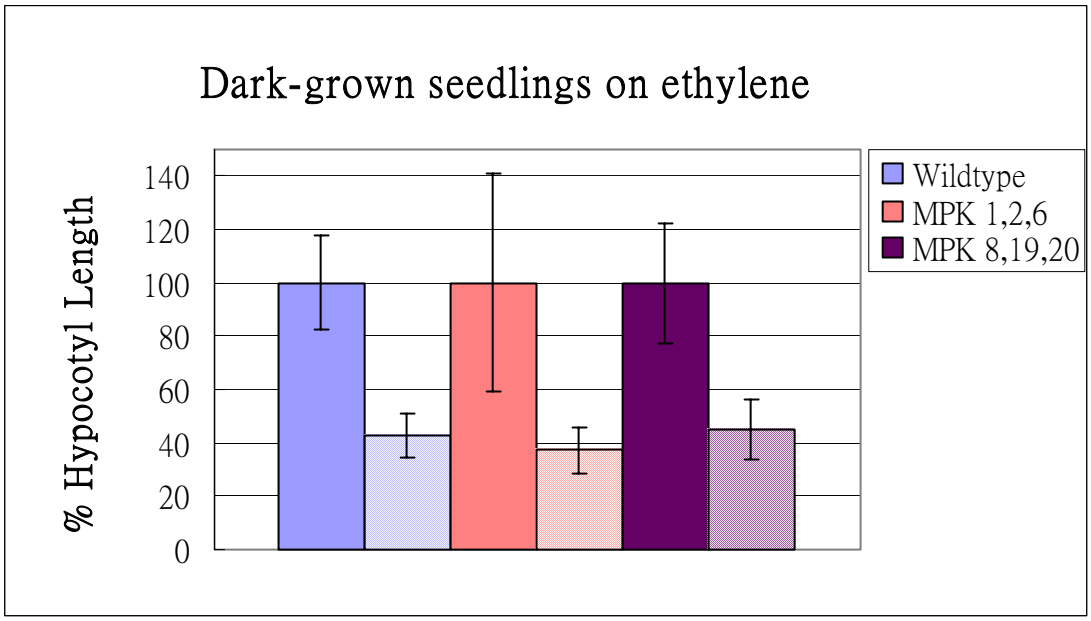

Figure 9. The hypocotyl growth reduction of wildtype and mutant seeds on ethylene. Wildtype, *MPK1,2,6* and *MPK8,19,20* mutant seeds were plated on ethylene and 0.5X MS media plates in the dark for 5-7 days after being exposed to light for 2 hours. The hypocotyl lengths of seeds grown on MS media were considered as 100% hypocotyl growth. The columns with solid colors represent the root growth of plants plated on MS media. The striped bars are the ethylene plates.

For the 14° C chill assay, the three types of seeds showed only 15-20% of root length compared to the control seedlings on MS media plates (Fig.10). The mutant seedlings showed no significant difference in root growth compared to the control seedlings.

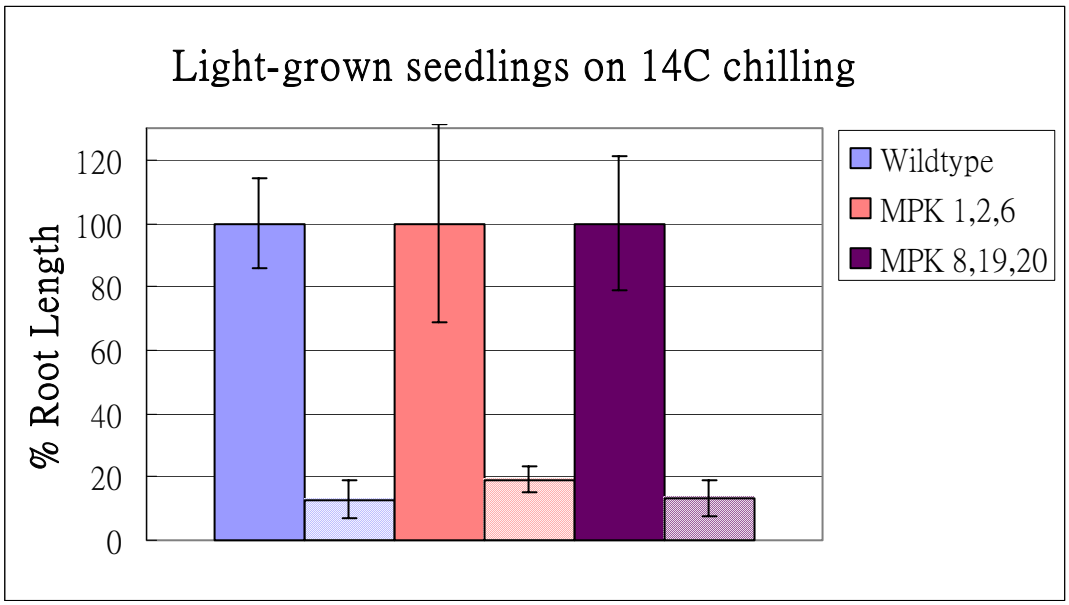

Figure 10. The root growth reduction of wildtype and mutant seeds in 14° chilling. Wildtype, *MPK1,2,6* and *MPK8,19,20* mutant seeds were plated on 0.5X MS media plates at 14° C and 21° C in the light for 5-7 days. The root lengths of seeds grown on MS media were considered as 100% root growth. The columns with solid colors represent the root growth of plants plated 21° C. The striped bars are the plates at 14° C.

Discussion

My hypothesis for this study was that *MPK1, 2* and *6* have similar functions in Arabidopsis and thus mutant plants would respond similarly under stress conditions. The same hypothesis was applied to *MPK8, 19* and *20*. These hypotheses were based on the data from the CSB Project on their transcriptional levels under stress conditions. However, my experiments showed that the *MPK1, 2, 6* and *MPK8, 19, 20* triple mutants had similar phenotypes as wildtype seedlings. Although *MPK1, 2* and *6* are not required in the stress response, so as *MPK8, 19* and *20*, they might still participate in the response pathways and the abnormal phenotype is masked by a wildtype *MPK* gene. In that case, we cannot conclude whether these genes are involved in the stress response. Hence, no conclusion can be made on whether my hypothesis was supported until abnormal phenotypes are observed in the mutants.

My experiments were not able to provide information on the functions of the six *MPK* genes studies. However, my approach of creating the desired mutants and comparing their phenotypes to those of wildtype plants provides an example of how reverse genetics can be used to study gene functions. For future study, the same experiments can be repeated in other experimental

conditions in hoping of abnormal phenotypes of mutants would be observed. By that time we can compare the phenotypes of the triple mutants to those of double and single mutants in order to figure out which gene or group of genes cause the phenotypic difference. Also, other combinations or higher levels of mutants can be created to study some other *MPK* genes. In conclusion, *MPK1, 2, 6* and *MPK 8, 19, 20* triple mutants show similar phenotypes as wildtype plants under the experimental conditions. The creation of target mutants in order to provide information on the knockout genes is a reverse genetic approach in understanding gene functions.

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