Role of MAPK phosphatase MKP2 and DsPTP1 in plant growth and development

Jianlei Sun

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science (Biology) at

Concordia University

Montreal, Quebec, Canada

April 2022

CONCORDIA UNIVERSITY
School of Graduate Studies

This is to certify that the thesis prepared

By: Jianlei Sun

Entitled: **Role of MAPK phosphatase MKP2 and DsPTP1 in plant growth and development**

and submitted in partial fulfillment of the requirements for the degree of

Master of Sciences (Biology)

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

_____ Chair
Dr. Selvadurai Dayanandan

_____ External Examiner
Dr. Gulick Patrick

_____ Examiner
Dr. Selvadurai Dayanandan

_____ Examiner
Dr. Madoka Gray-Mitsumune

_____ Supervisor
Dr. Jin Suk Lee

Approved by: _____
Robert Weladji, Chair of Department or Graduate Program Director

_____
Pascale Sicotte, Dean of Faculty of Arts and Science

Date April 14th 2022

Abstract

Role of MAPK phosphatase MKP2 and DsPTP1 in plant growth and development

Jianlei Sun, M.Sc

Mitogen-activated protein kinase (MAPK) cascades are a group of protein kinases composed of a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK that play an important role in signal transduction in response to various stimuli in eukaryotes. As terminal components of sequential phosphorylation events, the activity of MAPKs is upregulated through phosphorylation by upstream MAPKKs and downregulated through dephosphorylation catalyzed by protein phosphatases. In plants, MAPK cascades are relevant to a large number of biological processes, including plant growth, development, and stress responses.

The *Arabidopsis thaliana* genome contains five MAPK phosphatases (MKPs), MKP1, MKP2, DsPTP1, IBR5 and PHS1. Relatively little is known about their properties and biological roles, especially in plant growth and development compared to their substrate, MAPKs. Thus, our research group investigated the roles of these five MKPs in plant growth and development by analyzing single and higher-order *mkp* mutants. In this thesis, based on our original data obtained from these protein interaction researches, mutant studies as well as available data from the literature, we decided to focus on two *Arabidopsis* MKPs, MKP2 and DsPTP1, and their potential substrate MAPKs.

Neither *mkp2* nor *dsptp1* plants displayed obvious developmental phenotypes under standard growth conditions, but interestingly we found that *mkp2 dsptp1* double mutants resulted in a dramatic albino phenotype, eventually leading to *mkp2 dsptp1* seedling lethality. These results suggest that MKP2 and DsPTP1 play an important role in early plant growth and development.

To determine whether the albino phenotype of *mkp2 dsptp1* double mutants is caused by the loss of MKP2 and DsPTP1, transgenic *Arabidopsis* plants were constructed that have the expression of either *MKP2* or *DsPTP1* in the *mkp2 dsptp1* mutant background under its own native promoter. These complementation lines rescued the albino phenotype of *mkp2 dsptp1* mutants, indicating that the loss of *MKP2* and *DsPTP1* function is indeed responsible for the albino phenotype of *mkp2 dsptp1* mutants. It was observed that chloroplast number in guard cells on the epidermis and the

level of photosynthetic pigments were reduced significantly in *mkp2 dsptp1* when compared with either the wild-type or the complementation lines. These results suggest that MKP2 and DsPTP1 are functionally redundant novel regulators and are required for chloroplast development in *Arabidopsis.* To further understand how MKP2 and DsPTP1 integrate MAPK cascades in chloroplast biogenesis a search for their MAPK substrates was performed. Yeast two-hybrid assays confirmed our previous finding that MKP2 specifically interacts with two MAPKs, MPK8 and MPK15. This suggests that deregulation of MPK8 and/or MPK15 potentially leads to the dramatic albino phenotype with reduced chloroplasts in *mkp2 dsptp1*. Consistently, it was found that *mkp2 dsptp1 mpk8 mpk15* high-order mutants supressed the lethal seedling phenotype of *mkp2 dsptp1.* Based on this analyses, I propose that two MAPKs, MPK8 and MPK15 are potential substrates of MKP2 and DsPTP1 in chloroplast biogenesis. In summary, this work identified two MAPK phosphatases required for early plant development, chloroplast biogenesis, and also identified possible MAPK substrates for further study.

Acknowledgement

I would like to thank my committee members Dr. Selvadurai Dayanandan and Dr. Madoka Gray-Mitsumune as well as external examiner Dr. Patrick Gulick for their suggestions, patience, guidance and help.

I would like to thank all my lab members due to their cooperation, help and support in my scientific research. I really appreciate for making friends with them. With their encouragement, I made a big improvement in my research skills and significant progress in my research project.

I would like to thank Dr. Chris Law. Thanks for all his help in Confocal imaging and analysis, as well as his teachings in microscope equipment. I also want to acknowledge the support from Ms. Sonia Ruiz for her help in adjusting and repairing growth chambers. In addition, I am very grateful for the help which was provided by Mr. Kevin Moon and Mrs. Lyudmila Anohina, I want to express my appreciation to them for receiving and delivering critical chemicals to our lab.

I am truly thankful for the financial support provided by the Natural Sciences and Engineering Research Council of Canada, as well as the peaceful environment and advanced equipment that was provided by Concordia University.

I really appreciate my supervisor, Dr. Jin Suk Lee. She is enthusiastic, patient, knowledgeable and professional. In my two-year research experience, she provided warm help and professional guidance to me. Based on her guidance and teaching, I obtained scientific research training. This will be invaluable for my whole life. Her attitude of hard working affected me deeply and has become the lighthouse to guide my future life. Thank you Dr Lee, for encouraging me to make a huge progress in plant science.

Last but not least, I have great sense of gratitude to my parents. Because they gave me strong encouragement and support which has given me, a firm will and positive attitude which has allowed me to overcome difficulties during my research.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MKP	MAPK phosphatase
IBR5	Indole-3-butyric acid response 5
PHS1	Propyzamide-hypersensitive 1
DsPTP1	Dual specificity protein phosphatase 1
Col	Columbia
Y2H	Yeast two hybrid assay
ABA	Abscisic acid
CaM	Calmodulin
SAM	Shoot apical meristem
LHCB	Light-harvesting chlorophyll a/b-binding (LHCB) proteins
TOC	Translocon of outer membrane
TIC	Translocon of inner membrane
ROS	Reactive oxygen species
ABI4	ABA-INSENSITIVE (ABI) 4 factor
CAS	Calcium sensor protein
YFP	Yellow fluorescent protein
GFP	Green fluorescent protein
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
T-DNA	Transfer-DNA insertion
GBF	G-box-binding factor
CDS	Coding sequence
Ws	Wassilewskija
S	Serine

T	Threonine
X	Any amino acids
Y	Tyrosine
Chl	Chlorophyll

General Introduction

1.1. MAPK (Mitogen-activated protein kinase) signaling

Plants develop signaling pathways to receive external signals and then, adjusting physiological activities to avoid effects from environmental factors and maintain proper development, function and growth (He *et al*., 2020). MAPK cascades form one of the key components of signal transduction that regulates biological behaviors, such as proliferation, apoptosis, cellular survival and gene expression. In eukaryotes, the MAPK cascade consists of three critical components, MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAP kinase (MAPK) (Guo *et al*., 2020). The cascade receives environmental and/or internal signals and delivers them to substrates which activate biological activities, such as cellular proliferation, cellular differentiation, and hormonal responses (Cui *et al*., 2019). In response to an environmental cue, the activated MAPKKK delivers biological signals to the downstream MAPKK by phosphorylating serine or threonine residues in the S/T-X_{3-5}-S/T sequence (X can be any amino acid) of the MAPKK activation loop. MAPKK has phosphorylation sites with dual specificity on threonine and tyrosine residues in the T-X-Y motif in the activation loop of MAPK (Jiang and Chu, 2018). Activated MAPKs then phosphorylate various substrates, such as enzymes or transcription factors to regulate downstream biological activities.

Plants have many signaling pathways, and these pathways form a signal transduction network to regulate physiological activities. To perform complicated cellular functions and avoid chaos during cellular communication, the specificity is enriched in the MAPK pathway to avoid any unnecessary crosstalk between MAPKs and their respective substrates (Junttila *et al*. , 2008). Based on the *Arabidopsis thaliana* genome data, there are 80 MAPKKKs, 10 MAPKKs, and 20 MAPKs (Umezawa *et al*., 2011). In the MAPK phosphorylation cascade, MAPKK is the component with the lowest number. Ten MAPKKs phosphorylate 20 MAPKs, indicating that MAPKK is the integration point for many MAPK cascades (Plotnikov *et al*., 2011). This fact lends credence to the theory that MAPK signal transduction has a high level of specificity. Due to the high level of specificity, signal transmission rates from the receptor to the substrate are enhanced and the crosstalk between different pathways are avoided. Thus, the MAPK pathway plays an

essential role in improving the efficiency of many physiological responses triggered by various stimuli, such as temperature, salinity, drought, pathogen and insect infection (Bent, 2001).

Based on phylogenetic analysis of the MAPKs, they are divided into four groups. Group A has MAPK 3, 6, and 10. Members of Group B are MAPK 4, 5, and 11-13. Group C includes MAPK 1, 2, 7, and 14. Group D consists of MAPK 8, 9, and 15-20. Referring to the sequence similarity analysis, groups A, B, and C are the MAPK subgroups with the -TEY- motif. Group D MAPKs are notable in that they have the -TDY- instead of -TEY- motif in their activation loop (Jagodzik *et al*., 2018).

1.2. MAPK phosphatases

MAPKs are balanced by upstream phosphorylation from MAPKKs and negative regulation from MAPK phosphatases (Keyse, 2008). During phosphorylation, serine/threonine (ser/thr) and tyrosine (tyr) residues in the -TXY- motif within the activation loop of the MAPK are activated by the MAPKK. However, these activated residues are dephosphorylated by ser/thr phosphatase, tyr phosphatase and dual-specificity phosphatases. As the name implies, ser/thr phosphatase is the enzyme that cleaves phosphate from the ser and thr residues in proteins. Tyr phosphatase removes phosphate from tyr residues in the protein (Ruddraraju & Zhang., 2017). Dual-specificity phosphatase is a subgroup of tyr phosphatases; it specifically dephosphorylates activated tyr and ser/thr residues in the substrate (Brautigan， 2013； Zhang, 2002；Pulido & Lang, 2019). Some of these phosphatases are regarded as MAPK phosphatases.

MAPK proteins have two domains: the N-terminal half with the MAPK-binding domain and the C-terminal half with catalytical functions (Farooq *et al*., 2001). The N- and C-terminal MAPK-binding domains have high specificity due to the docking interaction between a negative amino acid residue and MKP that has positively charged amino acid residues (Tanoue *et al*., 2002). The structure of the dual-specificity domain changes after the activated MAPK binds to the MAPK-binding domain of MKP. The conformational change increases the efficiency of MAPK dephosphorylation (Farooq *et al*., 2001).

All MKPs have a specific homolog amino acid sequence in the dual-specificity domain. Their catalytic domains have a highly conserved consensus phosphatase sequence DX26(V/L)X(V/I)HCXAG(I/V)SRSXT(I/V)XXAY(L/I)M (X can be any amino acid) (Aoyama *et al*., 2001). There are 23 phosphatases identified in the *Arabidopsis* genome, but only five phosphatases have a MKP-unique AY[L/I]M motif found in mammalian MKPs (Jiang *et al*., 2018). These five *Arabidopsis* phosphatases are MAPK PHOSPHATASE 1 (MKP1), MAPK PHOSPHATASE2 (MKP2), DUAL SPECIFICITY PROTEIN PHOSPHATASE 1 (DsPTP1), PROPYZAMIDE-HYPERSENSITIVE (PHS1) and INDOLE-3-BUTYRIC ACID RESPONSE 5 (IBR5). In plants, there is a small number of MKPs but a large group of MAPK proteins, indicating that several MAPKs are likely regulated by a single MKP (Lee *et al*., 2008).

MKP1 is expressed widely in various plant tissues, and it is a stress and stimulus-inducible phosphatase that can bind to and dephosphorylate substrates in MAPK signaling (Toulouse & Nolan, 2015). The *mkp1* mutant in *Wassilewskija* (Ws) does not have distinct phenotypes compared to the wild-type (Ulm *et al*., 2001). However, the *mkp1* null mutant in Columbia (Col) has developmental defects, including early senescence, stomatal defects, and dwarfism. These abnormal phenotypes of the *mkp1* mutant in Col are due to the enhanced accumulation of salicylic acid. As well as that, the *mkp1* null mutant in Col leads to increased tolerance to salt stress (Bartels *et al.*, 2009). MKP1 has two calmodulin-binding domains. After the association with CaM, the dephosphorylation ability of MKP1 increases dramatically. This demonstrates that Ca^{2+} plays a role in MAPK signaling through the regulation of MKP1 (Lee *et al*., 2008). In yeast two hybrid assays, MKP1 shows physical interactions with stress-activated MPK3, MPK4, and MPK6 (Ulm *et al*., 2002). In addition, *mkp1* mutation enhances its resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pto*) and strengthens pathogen-associated molecular pattern (PAMP) -induced growth inhibition through regulation of MPK6 (Jiang *et al*., 2017).

PHS1 plays a crucial role in abscisic acid (ABA) signaling and microtubule formation (Tang *et al*., 2016). The *phs1-1* mutant displays a reduction in root length and twisted left-hand roots. The *phs1-3* mutant leads to ABA-triggered suppression of seed germination (Quettier *et al*., 2006). Based on yeast two-hybrid and BiFC assays, PHS1 physically interacts with MPK18. Additionally, the *mpk18* mutation results in the lack of microtubule-related functions. This indicates that MPK18

is a substrate of PHS1 and that the PHS1-MPK18 signaling module regulates microtubule functions (Walia *et al.*, 2009).

IBR5 is a phosphatase that regulates the auxin and ABA signaling pathways. In plants, the *ibr5* loss of function mutation leads to defects in the response to endogenous ABA and auxin (Monroe-Augustus *et al*., 2003). The *ibr5* mutant has a lower sensitivity to inhibition from exogenous ABA and auxin than the wild type. Based on yeast two-assays and *in vivo* protein co-immunoprecipitation studies, IBR5 physically interacts with the C-terminal domain of MPK12. The suppression of *MPK12* in *ibr5* complements the auxin-insensitivity in the *ibr5* single mutant. This indicates that MPK12 is a dephosphorylation substrate of IBR5, and that it positively regulates the auxin-related signaling pathway (Lee *et al*., 2009).

DsPTP1 is a dual-specific phosphatase that dephosphorylates and deactivates MPK4 *in vitro* (Gupta *et al*., 1998). In seed germination and seedling development, DsPTP1 is a negative regulator in osmotic stress signaling. Under osmotic stress, the *dsptp1* mutation causes a higher seed germination rate and longer root growth than the wild-type. Furthermore, under the same conditions, *DsPTP1* overexpression suppresses seed germination and root growth (Liu *et al*., 2015). Based on a gel overlay assay and site-directed mutagenesis, DsPTP1 has two Ca^{2+}-dependent CaM binding domains. The first one is on the N-terminal end of the protein and the second one is on the C-terminal end of the protein. With the increase of CaM concentration in *Arabidopsis*, the dephosphorylation activity of DsPTP1 on the *p*-nitrophenyl phosphate (pNPP) substrate increases. This indicates that the CaM-mediated Ca^{2+} signaling pathway regulates protein dephosphorylation by DsPTP1 (Yoo *et al*., 2004).

MKP2 is a protein that regulates responses to oxidative and pathogen-related stresses in *Arabidopsis*. Under ozone treatment, plants with suppressed *MKP2* expression showed prolonged phosphorylation of MPK3/6. This indicates that MPK3 and MPK6 are substrates of MKP2, and that this is an oxidatively related module (Lee & Ellis., 2007). In the aspect of plant immunity, MKP2 plays an essential role in plant-pathogen activities. In plants with a *mkp2* loss-of-function mutation, the immune responses to biotrophic pathogens are enhanced. Meanwhile, this mutation leads to weakened immune responses to the infection which is caused by necrotrophic pathogens.

Therefore, MPK3/6 and MKP2 form a MAPK network that mediates fungal elicitor-induced responses (Lumbreras *et al*., 2010).

1.3 Chloroplast and its Development

Chloroplast are organelles found in plants and alga and are essential for their growth, development and survival. Chloroplasts are the site of carbon dioxide fixation, production of photosynthetic pigments and synthesis of amino acids. One billion years ago, the chloroplast precursor was a symbiont to cyanobacteria. During evolution, chloroplasts have integrated into photosynthetic eukaryotic cells as an organelle for photosynthesis (Cavalier-Smith, 2002). In plants, chloroplasts develop from their precursors, proplastids, which are localized in meristematic cells (Pyke, 1999). During the development of plant cells, meristematic cells convert into mesophyll cells by differentiation and simultaneously, proplastids differentiate into chloroplasts (Yadav *et al.*, 2019).

Light is mandatory for the development of chloroplasts. Light exposure stimulates growth of cotyledons and chloroplasts, and suppresses hypocotyl development (Pogson *et al*., 2015; Rosa *et al*., 2020). As the shoot apical meristem (SAM) is activated to produce the leaf, at the same time, genes that are related to protein translation, cellular amplification, and chloroplast biogenesis are expressed (Barton, 2010; Sakamoto et al., 2008). In chloroplast development, the size of the proplastid expands dramatically, and the proplastid inner membrane differentiates into stroma by invagination (Lindquist *et al*.,2016). After this step, thylakoids appear in the stroma and form the stack by their amplification. Protein accumulation supports the growth of thylakoids from the cytosol. These proteins enter proplastids by protein import and are delivered to thylakoids by vesicles derived from the inner membrane of proplastids (Gutiérrez-Nava *et al*., 2004).

Thylakoids are a membrane system inside the chloroplast, and are the place where photosynthesis occurs. At the early developmental stage of chloroplasts, the precursor of the thylakoid is called the prothylakoid (Floris & Kühlbrandt, 2021). At that moment, enzymes that synthesize carotenoid and chlorophyll localize on the outer envelope membrane of proplastids. After pigments are synthesized, they are absorbed by light-harvesting chlorophyll-binding (LHCB) proteins when cytosolic proteins are transported into proplastids by protein import (Liu *et al*., 2013; Hobe *et al*.,

2000). Afterwards, pigment-protein complexes with chlorophyll, photosynthetic protein and enzymes are delivered to the prothylakoid by vesicles (Tominaga., 2018; Kubis *et al*., 2003). Then these pigment-protein complexes are absorbed by developing a thylakoid membrane for future thylakoid development.

During the development of thylakoid, many protein species are imported into proplastid by protein import from the cytosol. These proteins are identified and transported through an envelope of chloroplast via the TOC/TIC (translocon of outer/inner membrane) complex (Andrès *et al*., 2010). The TOC/TIC complexes are activated by light and exhibit strong specificity and selectivity to substrates. For example, the *TOC33* gene expression of young seedlings is upregulated by light. Then its protein import delivers and accumulates photosynthetic proteins in thylakoid. As well as that, TOC159 is highly specific to precursor protein which are related to photosynthesis in early chloroplast developmental stages (Kubis *et al*., 2004).

The acceleration in chloroplast division is a phenomenon that happens at the early differentiation of proplastids into chloroplasts. Similar to bacterial proliferation, binary fission is also used by chloroplast division. Binary fission is driven by a protein ring encoded by two genes, *FtsZ1* and *FtsZ2* (Yoshida, 2018; Chen *et al*., 2018). It indicates that nuclear-encoded genes for plastid ribosomal proteins are mainly expressed at early stages for proper chloroplast establishment, independent of plastid signals (Harrak *et al*., 1995). In contrast, nuclear-encoded photosynthetic genes are expressed at later stages of development because initiation of these genes is regulated by the retrograde communication from the well-developed chloroplast to the nucleus (Wang *et al*., 2020).

In the developing plant cells, coordination is required to balance communications between nuclear and plastid gene expression. There are two kinds of information exchange methods, anterograde (nucleus to plastids) and retrograde (plastids to the nucleus) communications (Grübler *et al*., 2017). Nucleus and plastid communications depend on biological signal motivations, and these signal molecules can be reactive oxygen species (ROS) or proteins (Mielecki *et al*., 2020). These two communications guarantee normal chloroplasts development and growth.

1.4 Photosynthetic pigments

In plants, there are two classes of photosynthetic pigments, the chlorophylls and carotenoids, which have the ability to absorb energy from sunlight and make it available to the photosynthetic apparatus. Chlorophyll a (Chla) absorbs energy from violet-blue and orange-red light, whereas chlorophyll b (Chlb) only absorbs the energy of blue and yellow light. (Elangovan & Murali, 2020; Myers & French,1960). Chla and Chlb are stabilized by light-harvesting Chla/b binding proteins at the antenna complex which transfers light energy to the reaction center containing Chla. During the light-dependent reaction of photosynthesis, the reaction center contributes light energy to the electron transport chain to support the synthesis of ATP and NADPH. These ATP and NADPH molecules are consumed in the dark reaction that produces glucose in chloroplasts (Allen, 2002; Laisk *et al*., 1991). Carotenoids not only have similar functions to Chla and Chlb, but also protect plants from photooxidation. This is because carotenoids degrade reactive oxygen species and convert excess energy to heat during photosynthesis (Sun *et al*., 2018).

1.5 MAPK signaling in chloroplast development

In *Arabidopsis thaliana*, the MAPK pathway participates in and regulates the development of chloroplasts. The MAPK pathway is found in the retrograde signaling network that coordinates gene expression of chloroplasts and the nucleus and maintains normal chloroplast biogenesis. In retrograde signaling (plastids to the nucleus), ABI4 is activated by phosphorylation from MPK3/6 to repress *LHCB* gene expression by binding to its promoter, maintaining normal photosynthetic activities in chloroplasts. MPK3/6 activities are initiated by upstream MKK4/5, which are activated by unknown MEKKs in the cascade. After association with 14-3-3ω protein, activity of the MKK4/5-MPK3/6 module is upregulated (Guo *et al.*, 2016). The level of association depends on the Ca^{2+} concentration in the cytosol in plant cells. A calcium sensor protein (CAS) that localizes on the thylakoid membrane detects extracellular Ca^{2+} concentration and light intensity. Under the effect of photoaccumulation, calcium stored in chloroplast binds to CAS, and they are then released to the cytosol to increase cytosolic Ca^{2+} concentration. Finally, the elevated Ca^{2+} concentration stimulates the phosphorylation of ABI4 through the 14-3-3ω-MKK4/5-MPK3/6 complex (Nomura and Shiina, 2014; Zhang *et al*., 2018). When the calcium sensor protein (CAS)

receives stimuli, chloroplasts release Ca^{2+} into cytosol. The increased cytosolic Ca^{2+} concentration improves the efficiency of the MAPK cascade by elevating its association with 14-3-3ω proteins. Simultaneously, the N-terminal fragment, which is produced from PTM (post-translational mechanism) enters the nucleus and activates *ABI4* expression. Activated MPK3/6 phosphorylates ABI4 to repress the expression of *LHCB* genes (Figure 1).

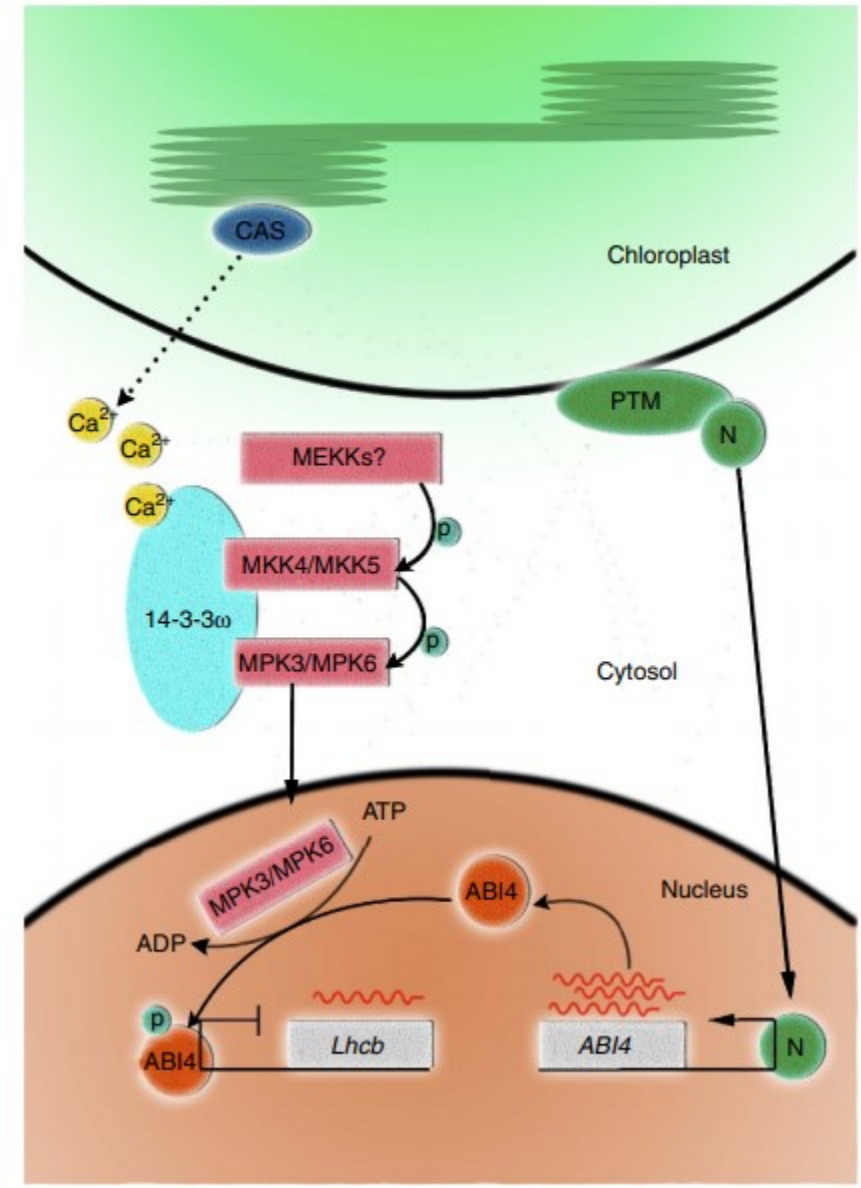

Figure 1. A model demonstrating the regulation of ABI4 activity by transcriptional and post-translational (MPK 3/6) mechanisms (Guo *et al*., 2016).

1.6 Thesis objective

MAPK signaling plays an important role in signal transduction in response to various extracellular stimuli in eukaryotes. The outcome of biological responses relies on the duration and amplitude of MAPK activity, which is regulated by the balance between upstream kinase (MKK) and phosphatase (MKP). Although regulation of MAPKs by upstream kinases has been recognized as important, little is known about their inactivation processes through the action of MKPs especially for proper plant growth and development. Therefore, one of the research objectives in the Lee Lab includes MKPs and their involvement in plant development. I took over an ongoing project about *MKP* genes and I had two main objectives: 1) Identify if any of the MKPs in *Arabidopsis* plays a role in controlling plant growth and development, and 2) discover their potential MAPK substrates that regulate plant development. Based on our initial mutant analysis for all five MKPs in *Arabidopsis*, we chose to study the role of two MAPK phosphatases, MKP2 and DsPTP1, in plant growth and development. Neither *mkp2* or *dsptp1* plants display obvious developmental phenotypes under standard growth conditions, but *mkp2 dsptp1* double mutants showed a dramatic albino phenotype, characterized by small and yellowish seedlings concomitantly with faster degradation of photosynthetic pigments. Based on these findings, all efforts were concentrated on the characterization of the role of MKP2 and DsPTP1 in chloroplast development and their potential MAPK substrates in *Arabidopsis*.

Materials and Methods

2.1 Plant materials and growth condition

mkp2-2 (SALK_045800), *dsptp1-1* (SALK_092811), *mpk8-1* (SALK_139288), and *mpk15-2* (SALK_061149) mutants were bought from The Arabidopsis Biological Resource Center (ABRC). All these mutants are in the *Arabidopsis thaliana Columbia* (Col) ecotype. To generate high-order mutants, single mutants were crossed and high-order mutants were identified by PCR genotyping in the F2 generation.

Seeds were sterilized in 1 ml of sterilization solution (33% bleach and 0.1% Triton in ddH_2O) for 10 minutes and were washed in sdd H_2O 5 times. The washed seeds were then sown on 1/2 Murashige and Skoog (MS) medium plates that had 1% sucrose and 0.35% agarose gel. The plates were then incubated at 4℃ for 3 days. Plates were moved to at 22℃ in a growth chamber (E15 Conviron) which was set to a 16hrs-light/8hrs-dark cycle. At 10 day postgermination (dpg), seedlings were transplanted to soil (2 black earth: 1 vermiculite: 1 peat moss).

2.2 DNA extraction, genotyping and PCR

A small piece of plant material was ground with a pestle in a microcentrifuge tube with 200µl of DNA extraction buffer (0.5% SDS, 250 mM NaCl, 200mM Tris-HCl pH7.5, 25 mM EDTA, and sddH_2O). The ground samples were centrifuged for 5 minutes at 13200 rpm. Then 150 µl of supernatant was transferred to a microcentrifuge and mixed with 150 µl of iso-propanol. Its incubation required at least 2 minutes. The microcentrifuge tube was centrifuged at 13300 rpm and its supernatant was discarded. Then 500µl of 95% ethanol was added into the microcentrifuge tube, then removed. Then the empty microcentrifuge tube was dried in the speed vacuum for 15-25 minutes. After drying, DNA on the wall of the microcentrifuge tube was resuspended by 30 µl of sddH_2O, and it was stored at 4℃. Samples were assayed by PCR reactions which were performed in Bio-Rad T100™ Thermal Cycler. Gene-specific primers were used to identify genotypes. Primer information is listed in Table 2.

2.3 Plasmid Construction

Constructs were generated and utilized in this research: pJSL28 (*MKP2 cDNA*), pJSL195 (*MKP2 promoter*), pJSL174 (*MKP2pro::MKP2*), pJSL242 (*MKP2* in pGADT7), pRJ38 (*DsPTP1 cDNA*) and pJSL196 (*DsPTP1 promoter*), pJSL192 (*DsPTP1pro::DsPTP1*), pJSL243 (*DsPTP1* in pGADT7), pJSL244 (*MPK8* in pGADT7), pJSL20 (*MPK8* cDNA), pJSL21 (*MPK15* cDNA), and pJSL245 (*MPK15* in pGADT7). All information about plasmid constructions in this research is listed in Table 1.

2.4 Agrobacterium transformation and generation of transgenic plants

Plasmid (2 μl) was transferred and mixed with *Agrobacterium tumefaciens* GV3101 strain competent cells . The mixture was kept on ice for 5 minutes. Then plasmid entered into agrobacteria in electric shock by Micro Pulser Electroporator (Bio-Rad). After that, 1 ml of SOC solution was added into the mixture and shaken at 28℃ for 1-1.5 hours at 200 rpm. Cells were spread on plates with LB media that has proper antibiotics for selection. Stable transformation of *Arabidopsis* plants was carried out using floral dip method. The overnight agrobacterium cell culture (400 μl) with constructs was transferred to 400 ml of LB media containing the appropriate antibiotics. Then this culture was incubated at 28℃ at 200rpm for at least 24 hours. After incubation, the culture was centrifuged at 6000 rpm for 17 minutes at 4℃ by Beckman Coulter high-performance centrifuges. The cell pellet was resuspended in 400 ml dipping solution (0.05% silwet and 5% sucrose in sddH_2O). The plant with 10-15 cm stem was dipped in the dipping solution with resuspended Agrobacterium twice for 5 seconds. In the next step, the plant were kept in the dark overnight. In the morning, the dipped plants was kept in the greenhouse and watered regularly. When the seed was set and plants were dried, seeds were harvested. After the collection of transgenic seeds from dried plants, seeds were sown on 1/2 Murashige and Skoog (MS) plate with antibiotics for selecting transformed plants. Seedlings with transformed with proper constructs thrived on the plates with antibiotics. They were transplanted to soil in pots and seeds of the next generation were collected.

2.5 Pigment quantification

Plant materials were frozen by liquid nitrogen and ground into powder. One hundred milligrams of powder were transferred into a microcentrifuge tube and suspend by 400µl of aqueous 80% acetone. The tubes were kept on ice at further steps. The tube was centrifuged at 4℃ at 13250 RPM. The supernatant was transferred into a new tube. Then, 200 µl of aqueous 80% acetone was added into the previous tube, it was vortexed and centrifuged again. The supernatant from the second extraction was added that of the first extraction. These steps were repeated until the pellet was white. The total volume of the extraction was noted and 100 µl of supernatant was transferred to a cuvette and then diluted by 900 µl of aqueous 80% acetone for estimating absorbance. The spectrometer was blanked at 750 nm with aqueous 80% acetone. Samples were measured at 470 nm, 646.6 nm, and 663.6 nm. Equations for calculating chlorophyll concentrations are followed:

[Chla] =12.25*E663.6-2.55*E646.6,

[Chlb] = 20.31* E646.6-4.91* E663.6,

[Chla+b] =17.76* E646.6+7.34* E663.6 ,

[Carotenoid] =(1000*E470-1.82*[Chla]-85.02*[Chlb])/198.

Units are in µg/ml at per 100 mg plant materials.

2.6 Yeast two-hybrid assay

Yeast cells (Y187 & AH109) were transferred to 10 ml of YPAD and was incubated at 28℃ overnight. The OD value should be 0.6. The 1 ml culture was centrifuged at 20000g, and the pellet was dissolved by ddH_2O. Simultaneously, PEG/LiOAc Master Mix solution (240 µl of 50%PEG, 36 µl of 1M LiOAc and 25 µl of salmon sperm DNA [10 mg/ml] for each combination). There are 0.5 µg of bait construct, 0.5 µg of prey construct, 300 µl of Master Mix solution and 100 µl of yeast cell suspension in a microcentrifuge tube as one combination. The microcentrifuge tube was heated at 42℃ for 45 minutes. In the next step, it was centrifuged at 850g, and the pellet was kept.

The pellet was then suspended in 200 µl of 0.9% NaCl. Yeast cells were spread on the SD-Leu/-Trp plate and were incubated at 28℃ for 48-72 hours. After the incubation, the colonies that grew on the plate were transferred to 100 µl of sddH_2O and was spread on the SD-Leu/-Trp/-Ura/-His plate. This plate was incubated for 4 days at 28°C.

2.7 Chloroplast assay

The Leica DM6000B epifluorescence microscope was used to observe the chloroplasts in stomatal guard cells on the abaxial side of leaf epidermis of *Arabidopsis* using a 63X objective lens. Before the visualization, plant material was dipped in 15% glycerol and placed on a glass slide. Chloroplasts images were captured by iPhone 6 via eyepiece.

2.8 Morphology of the wild-type and *mkp2 dsptp1* mutants grown under low light conditions

Wild-type and *mkp2 dsptp1* seedlings were grown in high/normal light (100-120 µE/m^2) and low light (5-8 µE/m^2) conditions with a life cycle of 16hrs light/8 hrs dark for 6 dpg in the growth chamber.

2.9 Tables

Table 1. Description of the plasmids used in this study.

Plasmid ID	Description	Insert	Vector	Bacterial Resistance	Plant Resistance
pJSL195	MKP2 promoter in pENTR5'-Topo TA	MKP2 promoter	pENTR5'-Topo TA	Kan	N/A
pJSL196	DsPTP1 promoter in pENTR5'-Topo TA	DsPTP1 promoter	pENTR5'-Topo TA	Kan	N/A
pJSL28	MKP2 in pKUT612	MKP2 cDNA	pKUT612	Kan	N/A
pJSL20	MPK8 in pKUT612	MPK8 cDNA	pKUT612	Kan	N/A
pJSL21	MPK15 in pKUT612	MPK15 cDNA	pKUT612	Kan	N/A
pRJ38	DsPTP1 in pKUT612	DsPTP1 cDNA	pKUT612	Kan	N/A
pJSL174	MKP2pro:MKP2	pJSL195	pGWB501 R4	Spec	Hyg
pJSL192	DsPTP1pro:DsPTP1	pJSL196	pGWB501 R4	Spec	Hyg
pJSL242	MKP2 in pGBKT7	pJSL28	pGBKT7	Amp	N/A
pJSL243	DsPTP1 in pGADT7	pRJ38	pGADT7	Amp	N/A
pJSL244	MPK8 in pGADT7	pJSL20	pGADT7	Amp	N/A
pJSL245	MPK15 in pGADT7	pJSL21	pGADT7	Amp	N/A

Table 2. The list of primers and their DNA sequences in this study.

Primer Names	DNA Sequence (from 5' to 3')
dsptp1-1 (SALK092811).f	TCCCTTCCCTTATTGAACAGG
dsptp1-1 (SALK092811).rc	AAACAATGACAGCCCATGAAC
DsPTP1 827f	GTGTTCTTGTTCATTGCTTTGTTGG
mkp2-2 (SALK045800) LP	TGTCTTAACCGTTGCTGTGG
mkp2-2 (SALK045800) RP	CTGGTTTGGGTATGGGATTG
mpk8-1 (SALK139288)f	GTGTTGTTGAGAAGACCAGCC
mpk8-1(SALK139288).rc	CTTCAAGATGAGCAAATTGCC
mpk15-2 (SALK061149)f	GGCTTCCAACTTCAGGTAAGC
mpk15-2 (SALK061149).rc	TCCAGCATCCAAGAATGAAAC
GFP.rc (GW)	TGCAGATGAACTTCAGGGTCAGCT
pGADT7.rc	TTATGTAGTCTGTTTTTTATGCAAAATCT
pGBKT7.rc	CCTGGCCTTTTGCTGGCCTTTTGCTCACA
LBa1	TGGTTCACGTAGTGGGCCATCG
LBb1.3	ATTTTGCCGATTTCGGAAC

Results

3.1 *Arabidopsis mkp2 dsptp1* mutants exhibit an albino phenotype

MKP2 and DsPTP1 are members of the MKP phosphatase family that has five members with unique AY [L/I]M motif in the *Arabidopsis* genome. T-DNA insertion mutants, *mkp2-2* (SALK_045800*)* and *dsptp1-1* (SALK_092811), were used for phenotypic analysis (Figure 2A) and the *Col* was used as a control. As our research group previously reported, either *mkp2-2* or *dsptp1-1* mutants do not have a significant phenotypical difference from the wild-type at 7 dpg (Figure 2B) (Tamnanloo *et al*., 2018). *Col*, *mkp2-2* and *dsptp1-1* seedlings were sown on the same 1/2 MS plate and transplanted at 10 dpg. Plants were kept in a growth chamber for 42 days at 80-100 μE/m^2. At 42 dpg, *Col*, *mkp2-2* and *dsptp1-1* mutants also showed identical phenotypes (Figure 2C).

In the phylogenetic tree analysis of the five MKPs that have unique AY [L/I]M motifs, it was found that MKP2 is the closest relative of DsPTP1 (Figure 3A). Moreover, in the comparison of the amino acid sequences of MKP2 and DsPTP1, it was found that they have high sequence identity and similarity; 64% for sequence identity, and 81% for sequence similarity (Figure 3B). Therefore, to reveal the potential functional redundancy of these two MKPs in *Arabidopsis*, our lab next generated *mkp2 dsptp1* double mutants by crossing *mkp2-2* (SALK_045800*)* and *dsptp1-1* (SALK_092811) mutants (Figure 4A). Interestingly, we found that unlike the single mutants, the *mkp2 dsptp1* double mutants showed a dramatic albino phenotype, characterized by small and yellowish seedlings (Figure 4B). The plants that were homozygous for *mkp2* and *dsptp1* only survived for roughly 10 days. In summary, our genetic analysis suggests functional redundancy of MKP2 and DsPTP1 in early plant growth and development in *Arabidopsis*.

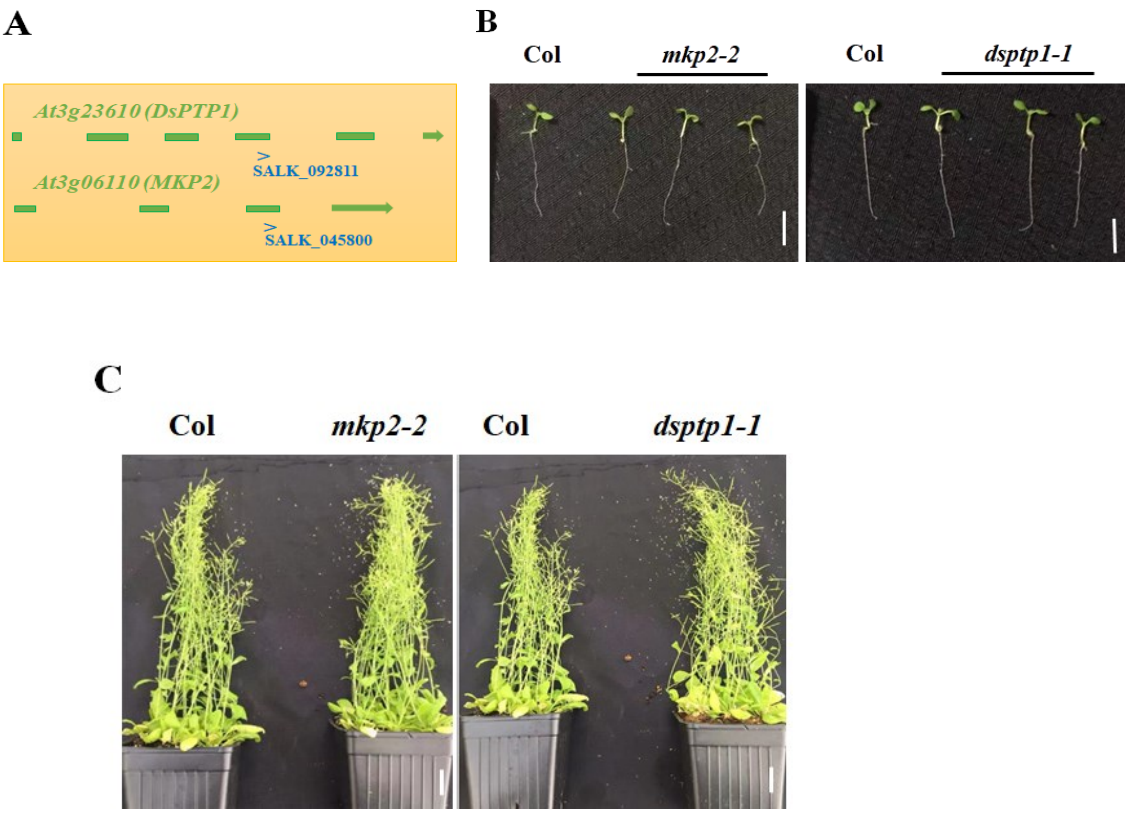

Figure 2. Identification of *mkp2-2* and *dsptp1-1* mutants.

(A) T-DNA insertion position of *mkp2-2* and *dsptp1-1*. The direction of the T-DNA was the same as the gene. The *dsptp1-1* and *mkp2-2* T-DNA insertion positions was indicated at T-DNA EXPRESS (http://signal.salk.edu/cgi-bin/tdnaexpress). Both of these mutants had T-DNA insertion in an exon.

(B) The growth phenotypes of 7 dpg seedlings of *mkp2-2* and *dsptp1-1* genotypes grown on 1/2 MS plates. Scale bar = 0.5 cm.

(C) The growth phenotypes of 42 dpg mature plants. Scale bar = 2 cm.

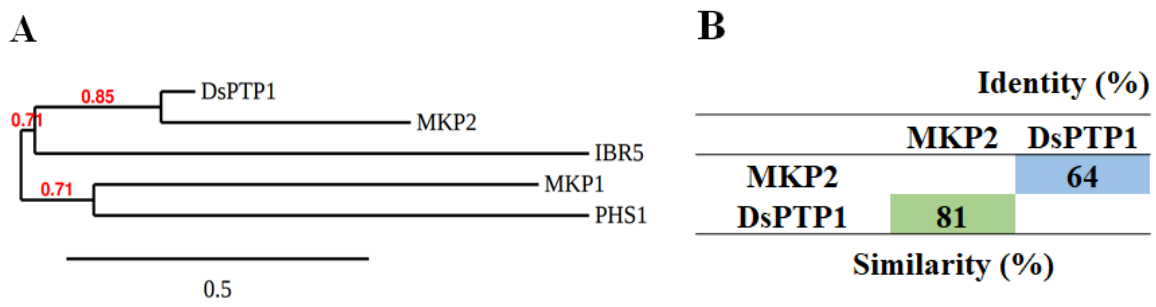

Figure 3. Phylogenetic analysis of MKP2 and DsPTP1, and amino acid identity and similarity.

(A) The phylogenetic tree of MKPs in *Arabidopsis*. The entire amino acid sequences of the MKPs were used for the phylogenetic analysis on phylogeny (www.phylogeny.fr). The phylogenetic tree was generated using the "One Click" mode with default settings, the bar indicates the branch length.

(B) Amino acid identity and similarity of MKP2 and DsPTP1. Full-length amino acid sequences of MKP2 and DsPTP1 were obtained from PHYTOZOME (https://phytozome.jgi.doe.gov/pz/portal.htmL) and subjected to identity and similarity assays on SIAS (http://imed.med.ucm.es/ Tools/sias.htmL). The blue section represents the amino acid sequence identity, and the green section represents the amino acid sequence similarity.

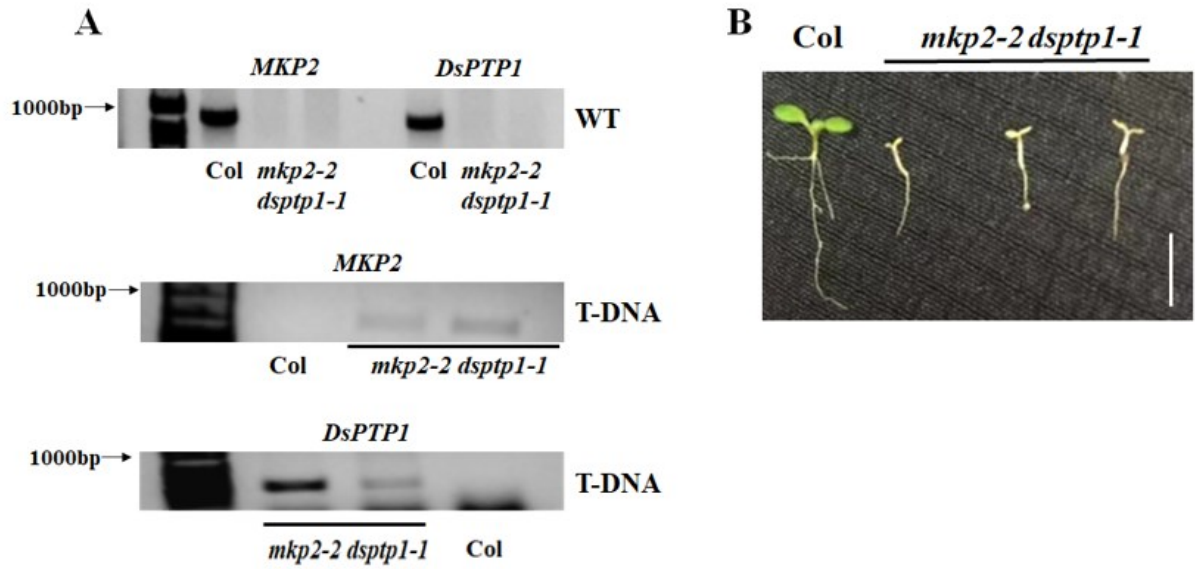

Figure 4. Identification and phenotypic analysis of the *mkp2 dsptp1* plants.

(A) Detection of *mkp2 dsptp1* double mutant by PCR genotyping. Genotyping primers mentioned in Table 2 were used to identify the homozygous *mkp2 dsptp1* double mutant. Homozygous *mkp2 dsptp1* double mutants did not produce a band because of the T-DNA insertion, whereas wild-type (*Col*) plants produced a band of ~ 1,000 bp in size.

(B) The seedling growth phenotypes of indicated genotypes (7 days post-germination) on 1/2 MS plates. Scale bar = 0.5 cm.

3.2 Complementation of *mkp2 dsptp1* loss-of-function mutants by *MKP2* or *DsPTP1*

To confirm whether the albino phenotype of the *mkp2 dsptp1* seedlings is due to the loss-of-function mutations of *MKP2* and *DsPTP1*, we next aimed to generate transgenic *Arabidopsis* plants expressing the full-length coding region of the *MKP2* or *DsPTP1* gene in the *mkp2 dsptp1* double mutant background under their own native promoters. Since the double mutants of *mkp2 dsptp1* are seedling lethal, *MKP2pro::MKP2* construct was introduced into *mkp2/+ dsptp1* and *DsPTP1pro::DsPTP1* construct was introduced into *mkp2 dsptp1/+*, respectively and then analyzed for whether the albino phenotype of *mkp2 dsptp1* is rescued. Homozygous transgenic plants expressing *MKP2* in the *mkp2/+ dsptp1* and expressing *DsPTP1* in the *mkp2 dsptp1/+* background were identified in the T3 generation. Unlike *mkp2/+ dsptp1* or *mkp2 dsptp1/+* mutants, one-quarter of these transgenic seedlings segregated as *mkp2 dsptp1* mutants did not show the albino and seedling lethal phenotype of *mkp2 dsptp1* double mutants. The genotype of these offspring was confirmed and *mkp2 dsptp1* homozygous plants from two independent T3 transgenic plants that carried fragments of *MKP2pro::MKP2* or *DsPTP1pro::DsPTP1* were obtained. As shown in Figure 5A and 5B, both *MKP2pro::MKP2* in *mkp2 dsptp1* and *DsPTP1pro::DsPTP1* in *mkp2 dsptp1* seedlings were indistinguishable from wild-type plants. In summary, these complementation analyses indicate that loss of MKP2 and DsPTP1 function is indeed responsible for the albino seedling phenotype of *mkp2 dsptp1* double mutants.

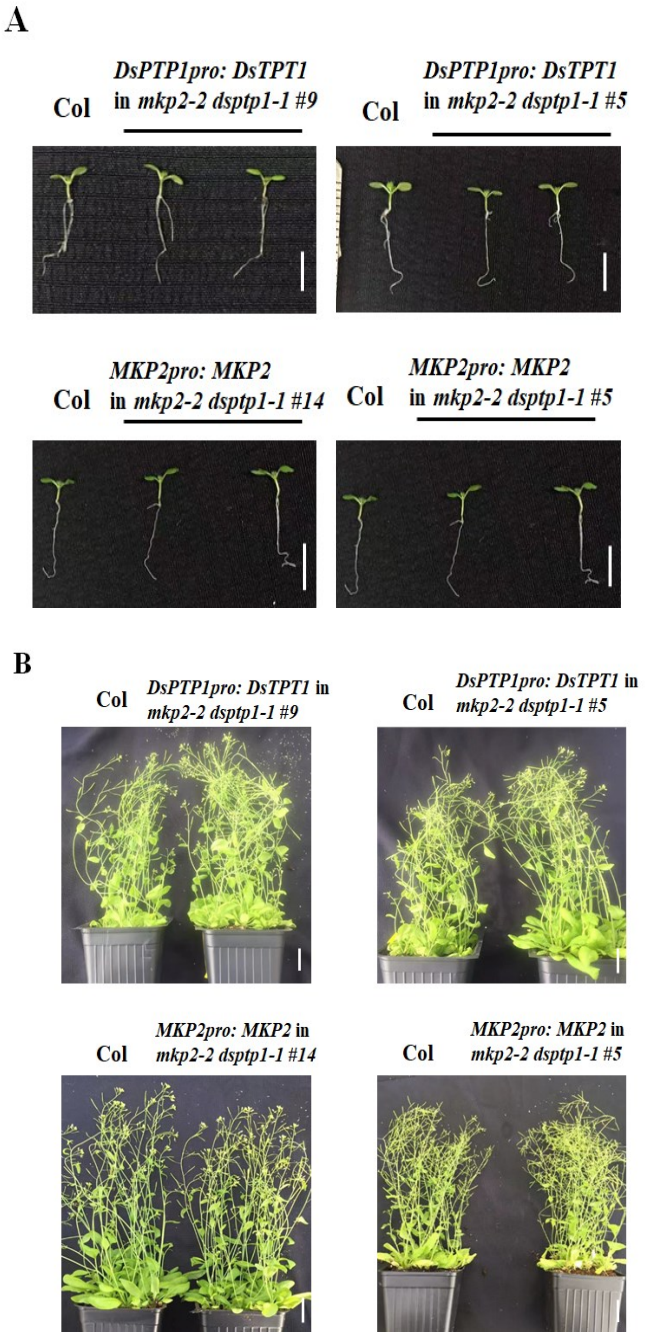

Figure 5. Complementation of the *mkp2 dsptp1* mutant.

(A) The seedling growth phenotypes of the indicated genotypes (7days post-germination) on 1/2 MS plates. Scale bar = 0.5 cm. *Col* was used as a control.

(B) The growth phenotypes of 42 dpg mature plants. Scale bar = 2 cm. *Col* was used as a control.

3.3 Chloroplast production is influenced by *MKP2* and *DsPTP1*

According to the results, *mkp2 dsptp1* plants display "white" cotyledons (Figure 4B) and *Col*, *MKP2* or *DsPTP1* complementation lines have green cotyledons (Figure 5A). The *mkp2 dsptp1* albino plants couldn't survive after the seedling stage, while *MKP2* or *DsPTP1* complementation lines grew well. Microscopic observations of *mkp2 dsptp1* seedlings, showed that their cotyledons were albino and transparent. Cotyledons of *Col* and complementation lines are green from the same stage seedlings (Figure 6A). This albino seedling phenotype of *mkp2 dsptp1* suggests that MKP2 and DsPTP1 possibly regulate generation of chloroplasts in *Arabidopsis.* Thus, chloroplast numbers in stomatal guard cells on the abaxial side of cotyledons in *Col*, complementation lines and *mkp2 dsptp1* mutants at three different ages (4, 7, and 10 dpg) (Figure 6) were examined. The average number of chloroplasts in guard cells of *Col*, two independent *MKP2pro::MKP2* in *mkp2 dsptp1* and *DsPTP1pro::DsPTP1* in *mkp2 dsptp1* plants are similar and stable from 4 dpg to 10 dpg. The average chloroplast number in these genotypes were around eight. However, *mkp2 dsptp1* has the lowest chloroplast number at 4 dpg among the genotypes examined, and it has a decreasing trend from 4 dpg to 10 dpg. At 10 dpg, some guard cells from *mkp2 dsptp1* averaged one and some cells didn't have any chloroplasts (Figure 6B). Altogether, *mkp2 dsptp1* mutants resulted in significantly decreased chloroplast development in stomatal guard cells. Although the number of chloroplasts was lower in the guard cells of the *mkp2 dsptp1* mutants, it was found that the size of these chloroplasts was not significantly different from those of wild-type as well as complementation lines tested. These results suggest that MKP2 and DsPTP1 play an important role in early chloroplast biogenesis in *Arabidopsis*.

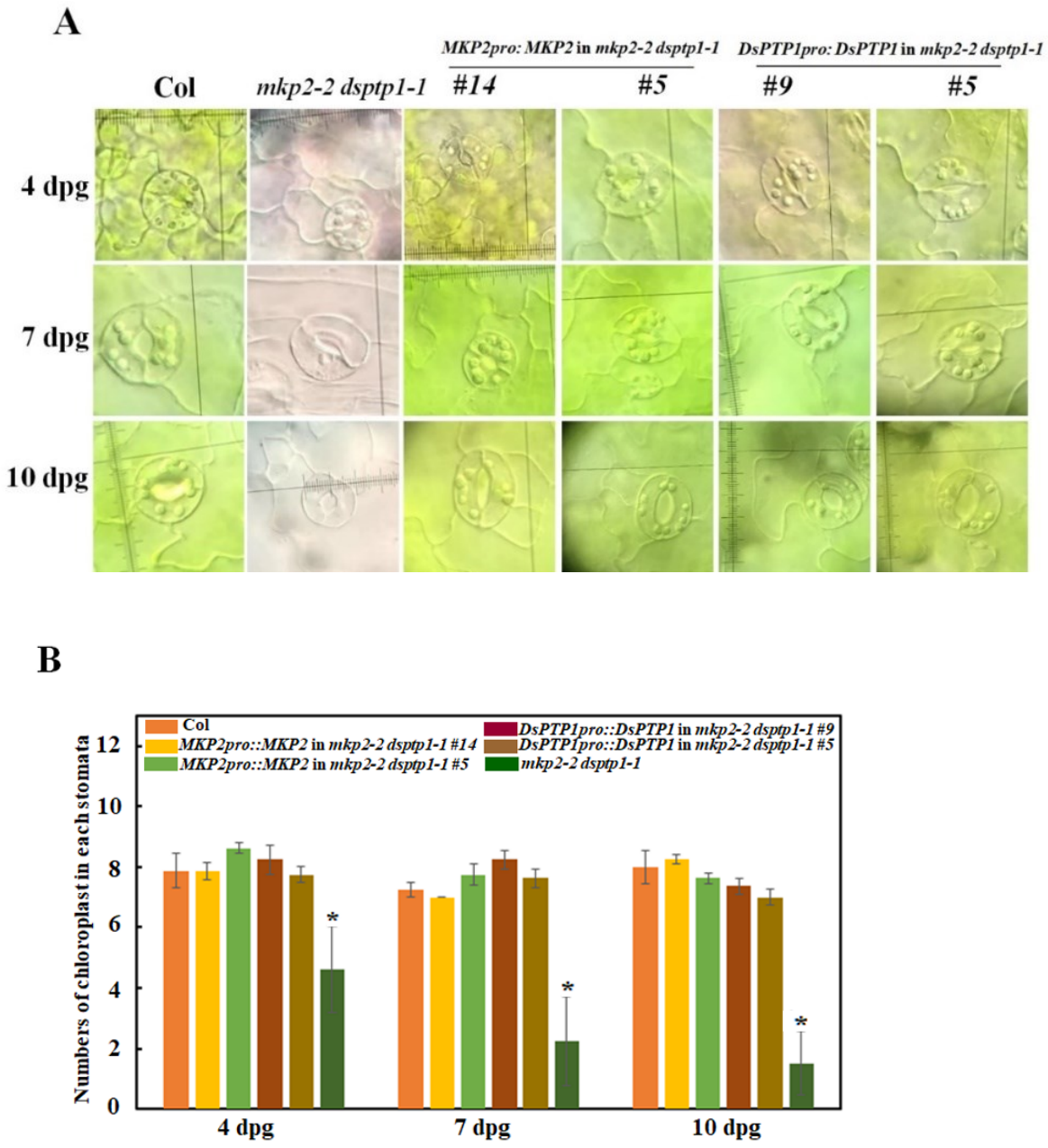

Figure 6. Chloroplast production in stomatal guard cells.

(A) Chloroplast production in Col, *mkp2 dsptp1*, and complementation lines. Pictures of 4 dpg, 7 dpg, 10 dpg stomata were taken with Leica DMI6000B epifluorescence microscope. Chloroplasts in than 10 stomata from different leaves were counted.

(B) The data represents the average ± standard deviation of 10 stomata at each stage. Seedlings were sown on 1/2 MS plates and grew in the same condition. Significant differences from *Col* at day 4, 7 and 10 are indicated (*$p < .05$).

3.4 The *mkp2 dsptp1* mutants exhibit a reduction in photosynthetic pigment levels

Since the level of photosynthetic pigments such as chlorophylls and carotenoids is one of the indicators of an albino phenotype, levels of photosynthetic pigments were measured in the *mkp2 dsptp1* mutants. Consistent with the albino seedling phenotype, the levels of chlorophylls and carotenoids were significantly lower in *mkp2 dsptp1* mutants than the wild-type and the complementation lines (*DsPTP1pro::DsPTP1* in *mkp2 dsptp1* and *MKP2pro::MKP2* in *mkp2 dsptp1*) (Figure 7).

The absence of carotenoid pigments can result in photo-oxidative stress under normal or high light conditions, which results in a secondary effect on chloroplast biogenesis. Furthermore, it is known that some of the photomorphogenic mutants exhibit albino phenotypes. Therefore, it was examined whether the homozygous progeny of *mkp2 dsptp1*/+ mutants develop the albino phenotype when they are grown under very dim light (approximately 5-8 $\mu E/m^2$). As shown in Figure 8, the albino phenotype of *mkp2 dsptp1* mutants is not changed under low light conditions, suggesting the albino phenotype of *mkp2 dsptp1* is not the consequence of photooxidative damage. This result also indicates that the major photomorphogenic mechanism such as de-etiolation (e.g. ceased hypocotyl elongation) is not affected in *mkp2 dsptp1*. Taken together, these observations suggest that MKP2 and DsPTP1 have a specific role for chloroplast development.

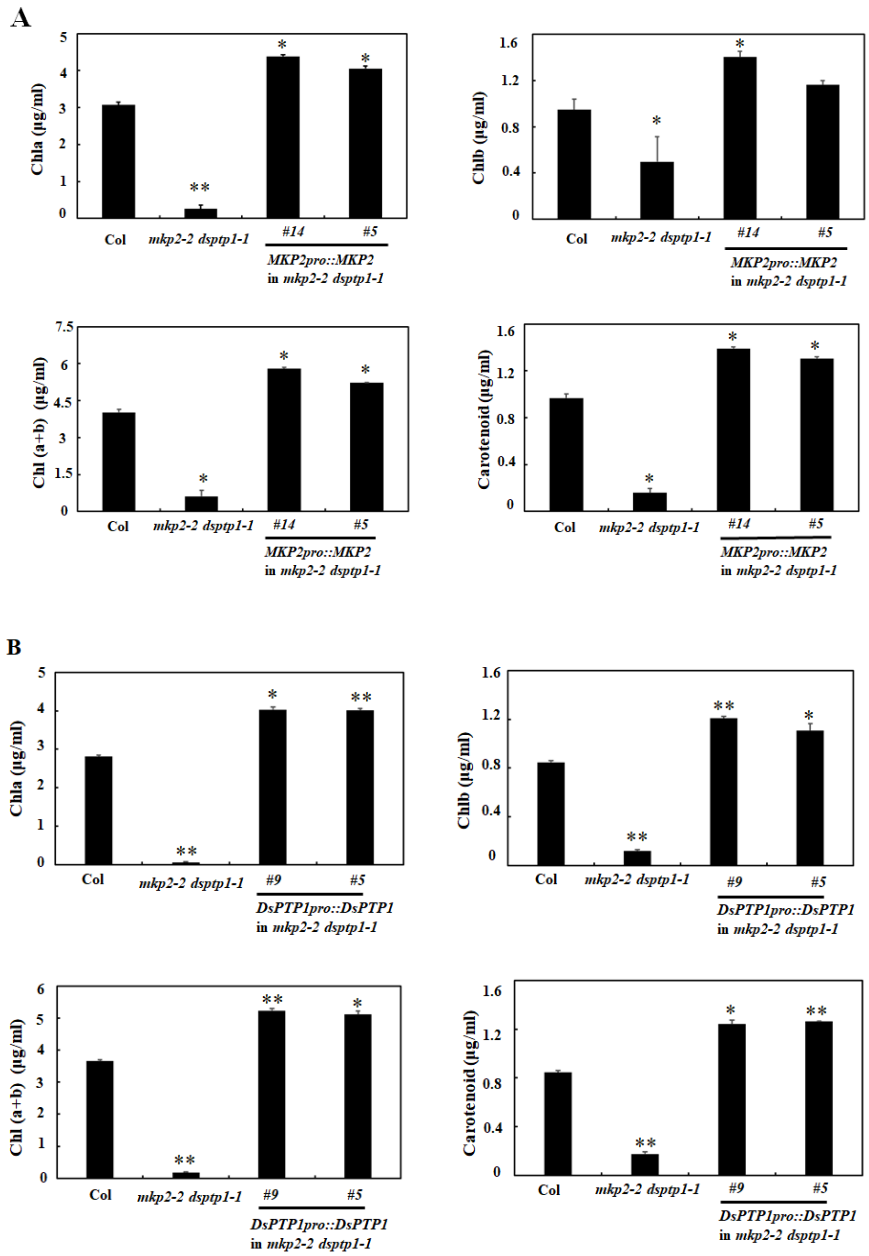

Figure 7. Content of chlorophyll and carotenoid pigments.

(A) and (B) The content of photosynthetic pigments was calculated at 6 days post-germination (dpg). Chla, chlorophyll a; Chlb, chlorophyll b; Chl (a+b), total chlorophyll. The data represents the mean ± standard deviation of three replicates. Significant differences from *Col* are indicated (*p < .05, **p<.0001).

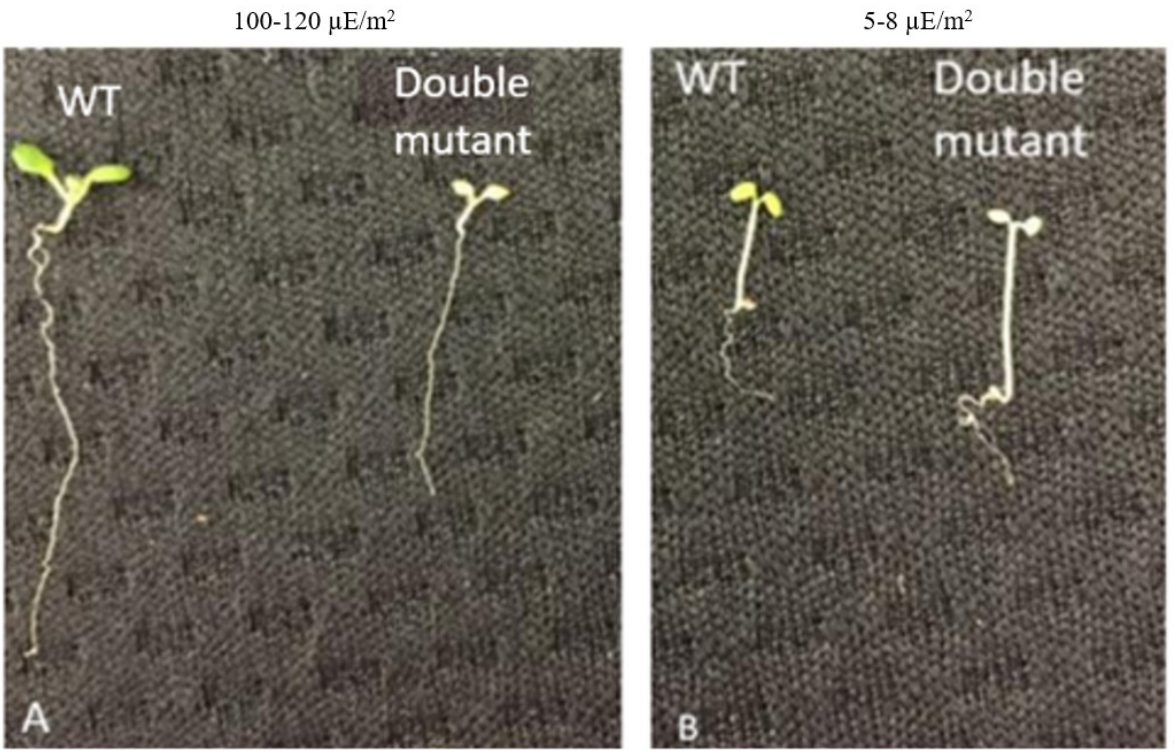

Figure 8. Morphology of wild-type and *mkp2 dsptp1* mutants grown under low light conditions.

(A) Wild-type (WT) and *mkp2 dsptp1* seedlings grown under high/normal light (100-120 µE/m^2) and (B) low light (5-8 µE/m^2) conditions. Seedlings were grown in 16hrs light for 6 days post-germination (dpg) under the light conditions indicated.

3.5 MKP2 interacts with MPK8 and MPK15

Twenty MAPKs are encoded in the *Arabidopsis* genome (Dóczi *et al*., 2007). Unlike the many members of the MAPK-family, there are only five MKPs including MKP2 and DsPTP1, which suggests that each of these MKPs could regulate multiple members of MAPKs.

To investigate potential MAPK substrate(s) of MKP2 and DsPTP1 in regulating chloroplast development, yeast two-hybrid analysis was performed to confirm our lab's previously finding that MPK8 and MPK15 are the MAPKs that physically interact with MKP2 in yeast. In each reaction, MKP2 was fused to the DNA-binding domain (BD) of GAL4, which was used as the bait protein. Meanwhile, *Arabidopsis* MPK8, MPK15 and DsPTP1 were fused to the activation domain (AD) as prey proteins. The bait and prey proteins were expressed simultaneously in yeast and were tested for interaction growth on a selection medium lacking histidine and uracil. As shown in Figure 9, on SD-Leu-Trp plates, all combinations had yeast growth, indicating that both plasmids were successfully transformed into the yeast strain. Besides, the yeast co-transformed with MKP2 bait fusion vector and MPK8/15 prey fusion vector also grew on plates containing SD -Leu/-Trp/-His/-Ura. However, yeast could not grow when co-transformed with the MKP2 bait fusion vector and DsPTP1 prey fusion vector are plated on SD -Leu/-Trp/-His/-Ura media plate. These results indicate that MKP2 specifically interacts with MPK8 and MPK15 in addition to MPK3 and MPK6, as previously reported.

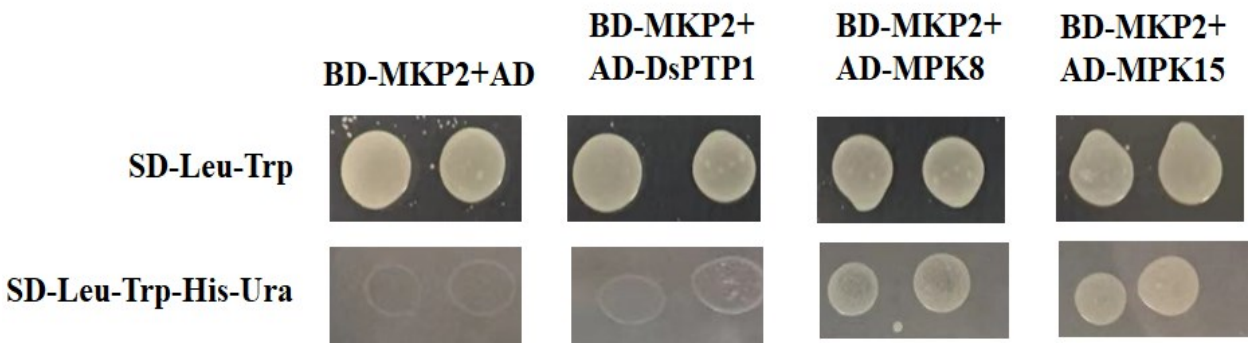

Figure 9. Yeast two-hybrid interactions between MKP2 and two specific MAPKs, MPK8 and MPK15.

Co-transformed bait and prey plasmids with AH109 yeast cells, and transformed yeast cells were grown on SD-Leu/-Trp plates for 3 days. The transformants contained both prey and bait plasmids. Transformants were recultured and plated on SD-Leu/-Trp/-His/-Ura plates for 4 days before pictures were taken.

3.6 MPK8 and MPK15 mediate *mkp2 dsptp1* developmental defects.

As mentioned above, the albino phenotype of the *mkp2 dsptp1* double mutant is due to defects in chloroplast development. Moreover, MKP2 showed physical interactions with MPK8 and MPK15. To find MAPK substrate(s) of MKP2 and DsPTP1 in regulating chloroplast biogenesis, higher-order mutants were created by crossing candidate MAPK mutant(s) with *mkp2 dsptp1*.

The interactions from the yeast two-hybrid assay indicate that MPK8 and MPK15 are underlying substrates of MKP2 and DsPTP1. Additionally, the chloroplast defects of *mkp2 dsptp1* offers some clues about the biological functions of these MAPKs in *Arabidopsis*. Thus, *mpk8-1 mpk15-2* double mutants were crossed with *mkp2 dsptp1*/+. At the F1 generation, plants that were hetero for T-DNA insertions in *MKP2, DsPTP1, MPK8, MPK15* were collected. At the F2 generation, *mkp2 dsptp1 mpk8-1 and mkp2 dsptp1 mpk15-2* homozygous triple mutants, as well as *mkp2 dsptp1 mpk8-1 mkp15-2* quadruple mutants after genotyping were obtained. As shown in Figure 10, unlike the albino and lethal seedling phenotype of *mkp2 dsptp1* mutants, *mpk8* and *mpk15* mutations suppressed the *mkp2 dsptp1* phenotype in the higher-order mutants created. This suggests that MPK8 and MPK15 may participate in the MAPK cascade in regulating chloroplast development.

Figure 10. MPK8 and MPK15 likely mediate *mkp2 dsptp1* developmental defects.

(A) The growth phenotypes of 7 dpg seedlings of (A) one wild-type and two *mkp2-2 dsptp1-1 mpk8-1* plants , (B) one wild-type and two *mkp2-2 dsptp1-1 mpk15-2* plants, and (C) one wild-type and two *mkp2-2 dsptp1-1 mpk8-1 mpk15-2* plants. Scale bar = 0.5 cm.

Discussion

4.1 Does MKP2 and DsPTP1 regulate chloroplast development?

The *Arabidopsis* genome has five MAPK phosphatases, MKP1, MKP2, DsPTP1, IBR5 and PHS1 and our research group previously found that none of five *mkp* single mutants displayed abnormal growth phenotypes compared to the wild-type plants. Thus, to examine the potential functional redundancy among these five MKPs, our group next generated different combinations of *mkp* higher-order mutants. Based on our genetic analysis, we found that unlike single and any of other double *mkp* mutants, *mkp2 dsptp1* double mutants displayed a dramatic albino phenotype, suggesting the defective of chlorophyll accumulation. chlorophyll content was evaluated and it was found that *mkp2 dsptp1* double mutants exhibit much lower chlorophyll levels than wild-type, while complementation line only with *MKP2pro::MKP2* or *DsPTP1pro::DsPTP1* in the *mkp2 dsptp1* background restores the wild-type phenotype (Figure 7). This demonstrates that no visible greening phenotype of *mkp2 dsptp1* mutants results from the loss of MKP2 and DsPTP1 and these two MKPs have functional redundancy in regulating chloroplast development and/or function.

As shown in Figure 7, we also found that the level of another photosynthetic pigment, carotenoids, in *mkp2-2 dsptp1-1* mutants is dramatically reduced. Carotenoids protect membrane structures of chloroplasts from the damage of photooxidation (Viljanen *et al*., 2002) and it has been reported that plants with reduced-carotenoid mutation experience more oxidative damage than wild-type plants (Ramel *et al*., 2013). Therefore, it was investigated if the albino phenotype of *mkp2 dsptp1* double mutants was the outcome of photooxidative stress due to the reduced levels of carotenoids. The albino, seedling lethal phenotype of *mkp2 dsptp1* mutants was not changed when they are grown under high/normal (100-120 μE) and low light (5-8 μE) conditions (Figure 8), which indicates that the albino phenotype of *mkp2 dsptp1* is not the result from photooxidation damage and likely caused by the direct defects in chloroplast biogenesis. Although there is a lack of detailed functional identification of MKP2 and DsPTP1 during different stages of chloroplast development, our chloroplast assay, the measurement of chlorophyll levels and phenotypic assays in low light conditions provide new functional information for MKP2 and DsPTP1 in controlling

chloroplast development. Transmission electron microscopy of *mkp2 dsptp1* mutants will further provide if these two MKPs influence chloroplast ultrastructure.

4.2 MPK8 and MPK15 are potential MAPK targets of MKP2 and DsPTP1 for the regulation of chloroplast development

The activity of MAPK, an important final determinant of biological response, is tightly regulated by a balance between the upstream kinase (MAPKK) and the corresponding phosphatase activity. Whereas MAPK activation relies on both phosphorylation of the ser/thr and tyr residues of the conserved TXY or TEY motif by the MAPKK, inactivation is mediated by dephosphorylation of either of these two residues (Jiang and Chu, 2018). Although it is well established that MKP, which is able to dephosphorylate both phosphoserine/threonine and phosphotyrosine residues of their downstream target proteins , play regulatory roles for MAPKs under various stress conditions, the MKP function and their substrate MAPKs in plant growth and development was relatively neglected.

After identification of two *Arabidopsis* MKPs, MKP2 and DsPTP1, having a role in controlling chloroplast development or function, I next performed yeast two-hybrid and genetic analyses to detect putative MAPK substrates. Our previous Y2H screen using all twenty *Arabidopsis* MAPKs (see also Figure 9) found that two MAPKs, MPK8 and MPK15, specifically bind to MKP2 while none of the MAPK proteins shows interaction with DsPTP1. Although the interaction between MPK8/15 and MKP2 needs to be verified *in planta*, our interaction data indicate that MPK8 and MPK15 are possible MAPK substrates of MKP2 and DsPTP1 and deregulation of these MAPKs might be the cause for the albino, seedling lethal phenotype of *mkp2 dsptp1*. Consistent with this idea, it was found that the high-order mutants, *mkp2 dsptp1 mpk8*, *mkp2 dsptp1 mpk15* and *mkp2 dsptp1 mpk8 mpk15* exhibit no abnormal developmental and growth phenotype. This suggests *mpk8* and *mpk15* mutation suppressed the albino phenotype of *mkp2 dsptp1*. While genetic analysis clearly support MPK8 and MPK15 as the substrates of MKP2 and DsPTP1, detailed various phenotypic analyses of higher-order mutants compared to the phenotype of *mkp2 dsptp1* mutants remain to be elucidated. These chloroplast-related phenotypic analyses will clarify if MPK8 and MPK15 have partially redundant function in controlling chloroplast biogenesis.

It has been reported that other Arabidopsis MAPKs, MPK3 and MPK6, are the substrates of MKP2 and they serve as important regulators of oxidative and biotic stress signaling (Lee & Ellis., 2007; Lumbreras *et al*., 2010). Although no biological evidence is reported, MPK4 is also shown to be dephosphorylated by DsPTP1 *in vitro* (Gupta *et al*., 1998). These studies suggest that multiple MAPKs can be regulated by the same phosphatases, MKP2 and DsPTP1, and it is possible any of these MAPKs also has a role in chloroplast biogenesis.

Reference

Aoyama, K., Nagata, M., Oshima, K., Matsuda, T., & Aoki, N. (2001). Molecular cloning and characterization of a novel dual specificity phosphatase, LMW-DSP2, that lacks the cdc25 homology domain. *J Biol Chem.*, *276*(29), 27575-27583.

Andrès, C., Agne, B., & Kessler, F. (2010). The TOC complex: preprotein gateway to the chloroplast. *Biochim Biophys Acta.*, *1803*(6), 715-723.

Allen, J. F. (2002). Photosynthesis of ATP—electrons, proton pumps, rotors, and poise. *Cell*, *110*(3), 273-276.

Bent, A. F. (2001). Plant mitogen-activated protein kinase cascades: negative regulatory roles turn out positive. *Proc Natl Acad Sci USA.*, *98*(3), 784-786.

Brautigan, D. L. (2013). Protein Ser/Thr phosphatases–the ugly ducklings of cell signalling. *FEBS J.*, *280*(2), 324-325.

Barton, M. K. (2010). Twenty years on: the inner workings of the shoot apical meristem, a developmental dynamo. *Dev Biol.*, *341*(1), 95-113.

Bartels, S., Anderson, J. C., Besteiro, M. A. G., Carreri, A., Hirt, H., Buchala, A., ... & Ulm, R. (2009). MAP kinase phosphatase1 and protein tyrosine phosphatase1 are repressors of salicylic acid synthesis and SNC1-mediated responses in Arabidopsis. *Plant Cell.*, *21*(9), 2884-2897.

Cavalier-Smith, T. (2002). Chloroplast evolution: secondary symbiogenesis and multiple losses. *Curr Biol.*, *12*(2), R62-R64.

Cui, L., Yang, G., Yan, J., Pan, Y., & Nie, X. (2019). Genome-wide identification, expression profiles and regulatory network of MAPK cascade gene family in barley. *BMC genomics*, *20*(1), 1-20.

Chen, C., MacCready, J. S., Ducat, D. C., & Osteryoung, K. W. (2018). The molecular machinery of chloroplast division. *Plant Physiol.*, *176*(1), 138-151.

Dóczi, R., Brader, G., Pettkó-Szandtner, A., Rajh, I., Djamei, A., Pitzschke, A., ... & 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*(10), 3266-3279.

de la Luz Gutiérrez-Nava, M., Gillmor, C. S., Jiménez, L. F., Guevara-García, A., & León, P. (2004). CHLOROPLAST BIOGENESIS genes act cell and noncell autonomously in early chloroplast development. *Plant Physiol.*, *135*(1), 471-482.

Elangovan, A., & Murali, V. (2020). Mapping the chlorophyll-a concentrations in hypereutrophic Krishnagiri Reservoir (India) using Landsat 8 Operational Land Imager. *Lakes & Reservoirs: Research & Management*, *25*(4), 377-387.

Floris, D., & Kühlbrandt, W. (2021). Molecular landscape of etioplast inner membranes in higher plants. *Nat plants.*, *7*(4), 514-523.

Farooq, A., Chaturvedi, G., Mujtaba, S., Plotnikova, O., Zeng, L., Dhalluin, C., ... & Zhou, M. M. (2001). Solution structure of ERK2 binding domain of MAPK phosphatase MKP-3: structural insights into MKP-3 activation by ERK2. *Mol cell.*, *7*(2), 387-399.

Guo, Y. J., Pan, W. W., Liu, S. B., Shen, Z. F., Xu, Y., & Hu, L. L. (2020). ERK/MAPK signalling pathway and tumorigenesis. *Exp Ther Med.*, *19*(3), 1997-2007.

Gupta, R., Huang, Y., Kieber, J., & Luan, S. (1998). Identification of a dual-specificity protein phosphatase that inactivates a MAP kinase from Arabidopsis. *Plant J.*, *16*(5), 581-589.

Grübler, B., Merendino, L., Twardziok, S. O., Mininno, M., Allorent, G., Chevalier, F., ... & Pfannschmidt, T. (2017). Light and plastid signals regulate different sets of genes in the albino mutant pap7-1. *Plant Physiol.*, *175*(3), 1203-1219.

Guo, H., Feng, P., Chi, W., Sun, X., Xu, X., Li, Y., ... & Zhang, L. (2016). Plastid-nucleus communication involves calcium-modulated MAPK signalling. *Nat Commun.*, *7*(1), 1-15.

He, X., Wang, C., Wang, H., Li, L., & Wang, C. (2020). The function of MAPK cascades in response to various stresses in horticultural plants. *Front Plant Sci.*, *11*, 952.

Harrak, H., Lagrange, T., Bisanz-Seyer, C., Lerbs-Mache, S., & Mache, R. (1995). The expression of nuclear genes encoding plastid ribosomal proteins precedes the expression of chloroplast genes during early phases of chloroplast development. *Plant physiol.*, *108*(2), 685-692.

Hobe, S., Niemeier, H., Bender, A., & Paulsen, H. (2000). Carotenoid binding sites in LHCIIb: Relative affinities towards major xanthophylls of higher plants. *Eur J Biochem.*, *267*(2), 616-624.

Hong, Y., Liu, Q., Cao, Y., Zhang, Y., Chen, D., Lou, X., ... & Cao, L. (2019). The OsMPK15 negatively regulates Magnaporthe oryza and Xoo disease resistance via SA and JA signaling pathway in rice. *Front Plant Sci.*, *10*, 752.

Jiang, M., & Chu, Z. (2018). Comparative analysis of plant MKK gene family reveals novel expansion mechanism of the members and sheds new light on functional conservation. *BMC Genomics*, *19*(1), 1-18.

Junttila, M. R., Li, S. P., & Westermarck, J. (2008). Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *The FASEB J.*, *22*(4), 954-965.

Jagodzik, P., Tajdel-Zielinska, M., Ciesla, A., Marczak, M., & Ludwikow, A. (2018). Mitogen-activated protein kinase cascades in plant hormone signaling. *Front Plant Sci.*, *9*, 1387.

Jiang, L., Chen, Y., Luo, L., & Peck, S. C. (2018). Central roles and regulatory mechanisms of dual-specificity MAPK phosphatases in developmental and stress signaling. *Front Plant Sci.*, *9*, 1697.

Jiang, L., Anderson, J. C., Besteiro, M. A. G., & Peck, S. C. (2017). Phosphorylation of Arabidopsis MAP kinase phosphatase 1 (MKP1) is required for PAMP responses and resistance against bacteria. *Plant Physiol.*, *175*(4), 1839-1852.

Keyse, S. M. (2008). Dual-specificity MAP kinase phosphatases (MKPs) and cancer. *Cancer Metastasis Rev.*, *27*(2), 253-261.

Kubis, S., Baldwin, A., Patel, R., Razzaq, A., Dupree, P., Lilley, K., ... & Jarvis, P. (2003). The Arabidopsis ppi1 mutant is specifically defective in the expression, chloroplast import, and accumulation of photosynthetic proteins. *Plant Cell.*, *15*(8), 1859-1871.

Kubis, S., Patel, R., Combe, J., Bédard, J., Kovacheva, S., Lilley, K., ... & Jarvis, P. (2004). Functional specialization amongst the Arabidopsis Toc159 family of chloroplast protein import receptors. *Plant Cell.*, *16*(8), 2059-2077.

Lee, J. S., Wang, S., Sritubtim, S., Chen, J. G., & Ellis, B. E. (2009). Arabidopsis mitogen-activated protein kinase MPK12 interacts with the MAPK phosphatase IBR5 and regulates auxin signaling. *Plant J.*, *57*(6), 975-985.

Lee, K., Song, E. H., Kim, H. S., Yoo, J. H., Han, H. J., Jung, M. S., ... & Chung, W. S. (2008). Regulation of MAPK phosphatase 1 (AtMKP1) by calmodulin in Arabidopsis. *J Biol Chem.*, *283*(35), 23581-23588.

Liu, R., Liu, Y., Ye, N., Zhu, G., Chen, M., Jia, L., ... & Zhang, J. (2015). AtDsPTP1 acts as a negative regulator in osmotic stress signalling during Arabidopsis seed germination and seedling establishment. *J Exp Bot.*, *66*(5), 1339-1353.

Lumbreras, V., Vilela, B., Irar, S., Solé, M., Capellades, M., Valls, M., ... & Pagès, M. (2010). MAPK phosphatase MKP2 mediates disease responses in Arabidopsis and functionally interacts with MPK3 and MPK6. *Plant J.*, *63*(6), 1017-1030.

Liu, R., Xu, Y. H., Jiang, S. C., Lu, K., Lu, Y. F., Feng, X. J., ... & Zhang, D. P. (2013). Light-harvesting chlorophyll a/b-binding proteins, positively involved in abscisic acid signalling, require a transcription repressor, WRKY40, to balance their function. *J Exp Bot.*, *64*(18), 5443-5456.

Lee, J. S., & Ellis, B. E. (2007). Arabidopsis MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. *J Biol Chem.*, *282*(34), 25020-25029.

Lindquist, E., Solymosi, K., & Aronsson, H. (2016). Vesicles are persistent features of different plastids. *Traffic.*, *17*(10), 1125-1138.

Laisk, A., Siebke, K., Gerst, U., Eichelmann, H., Oja, V., & Heber, U. (1991). Oscillations in photosynthesis are initiated and supported by imbalances in the supply of ATP and NADPH to the Calvin cycle. *Planta*, *185*(4), 554-562.

Monroe-Augustus, M., Zolman, B. K., & Bartel, B. (2003). IBR5, a dual-specificity phosphatase-like protein modulating auxin and abscisic acid responsiveness in Arabidopsis. *Plant Cell.*, *15*(12), 2979-2991.

Myers, J., & French, C. S. (1960). Evidences from action spectra for a specific participation of chlorophyll b in photosynthesis. *J Gen Physiol.*, *43*(4), 723-736.

Mielecki, J., Gawroński, P., & Karpiński, S. (2020). Retrograde signaling: Understanding the communication between organelles. *Int J Mol Sci.*, *21*(17), 6173.

Nomura, H., & Shiina, T. (2014). Calcium signaling in plant endosymbiotic organelles: mechanism and role in physiology. *Mol Plant.*, *7*(7), 1094-1104.

Plotnikov, A., Zehorai, E., Procaccia, S., & Seger, R. (2011). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys Acta.*, *1813*(9), 1619-1633.

Pulido, R., & Lang, R. (2019). Dual specificity phosphatases: from molecular mechanisms to biological function. *Int J Mol Sci.*, *20*(18), 4372.

Pyke, K. A. (1999). Plastid division and development. *Plant Cell.*, *11*(4), 549-556.

Pogson, B. J., Ganguly, D., & Albrecht-Borth, V. (2015). Insights into chloroplast biogenesis and development. *Biochim Biophys Acta.*, *1847*(9), 1017-1024.

Pipitone, R., Eicke, S., Pfister, B., Glauser, G., Falconet, D., Uwizeye, C., ... & Demarsy, E. (2020). Two distinct phases of chloroplast biogenesis during de-etiolation in Arabidopsis thaliana. *bioRxiv*.

Quettier, A. L., Bertrand, C., Habricot, Y., Miginiac, E., Agnes, C., Jeannette, E., & Maldiney, R. (2006). The phs1-3 mutation in a putative dual-specificity protein tyrosine phosphatase gene

provokes hypersensitive responses to abscisic acid in Arabidopsis thaliana. *Plant J.*, *47*(5), 711-719.

Ruddraraju, K. V., & Zhang, Z. Y. (2017). Covalent inhibition of protein tyrosine phosphatases. *Mol BioSyst.*, *13*(7), 1257-1279.

Ramel, F., Mialoundama, A. S., & Havaux, M. (2013). Nonenzymic carotenoid oxidation and photooxidative stress signalling in plants. *J Exp Bot.*, *64*(3), 799-805.

Sun, T., Yuan, H., Cao, H., Yazdani, M., Tadmor, Y., & Li, L. (2018). Carotenoid metabolism in plants: the role of plastids. *Mol Plant.*, *11*(1), 58-74.

Sakamoto, W., Miyagishima, S. Y., & Jarvis, P. (2008). Chloroplast biogenesis: control of plastid development, protein import, division and inheritance. *Arabidopsis book.*, *6*.

Tominaga, J., Nakahara, Y., Horikawa, D., Tanaka, A., Kondo, M., Kamei, Y., ... & Shimada, H. (2018). Overexpression of the protein disulfide isomerase AtCYO1 in chloroplasts slows dark-induced senescence in Arabidopsis. *BMC Plant Biol.*, *18*(1), 1-9.

Tang, Q., Guittard-Crilat, E., Maldiney, R., Habricot, Y., Miginiac, E., Bouly, J. P., & Lebreton, S. (2016). The mitogen-activated protein kinase phosphatase PHS1 regulates flowering in Arabidopsis thaliana. *Planta*, *243*(4), 909-923.

Tamnanloo, F., Damen, H., Jangra, R., & Lee, J. S. (2018). MAP KINASE PHOSPHATASE1 controls cell fate transition during stomatal development. *Plant Physiol.*, *178*(1), 247-257.

Tanoue, T., Yamamoto, T., & Nishida, E. (2002). Modular structure of a docking surface on MAPK phosphatases. *J Biol Chem.*, *277*(25), 22942-22949.

Toulouse, A., & Nolan, Y. M. (2015). A role for mitogen-activated protein kinase phosphatase 1 (MKP1) in neural cell development and survival. *Neural Regen Res.*, *10*(11), 1748.

Umezawa, T., Hirayama, T., Kuromori, T., & Shinozaki, K. (2011). The regulatory networks of plant responses to abscisic acid. *Adv Bot Res.*, *57*, 201-248.

Ulm, R., Revenkova, E., di Sansebastiano, G. P., Bechtold, N., & Paszkowski, J. (2001). Mitogen-activated protein kinase phosphatase is required for genotoxic stress relief in Arabidopsis. *Genes Dev.*, *15*(6), 699-709.

Ulm, R., Ichimura, K., Mizoguchi, T., Peck, S. C., Zhu, T., Wang, X., ... & Paszkowski, J. (2002). Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. *The EMBO J.*, *21*(23), 6483-6493.

Viljanen, K., Sundberg, S., Ohshima, T., & Heinonen, M. (2002). Carotenoids as antioxidants to prevent photooxidation. *Eur J Lipid Sci and Technol.*, *104*(6), 353-359.

Walia, A., Lee, J. S., Wasteneys, G., & Ellis, B. (2009). Arabidopsis mitogen-activated protein kinase MPK18 mediates cortical microtubule functions in plant cells. *Plant J.*, *59*(4), 565-575.

Wang, L., Leister, D., & Kleine, T. (2020). Chloroplast development and genomes uncoupled signaling are independent of the RNA-directed DNA methylation pathway. *Sci Rep.*, *10*(1), 1-12.

Yoshida, Y. (2018). Insights into the mechanisms of chloroplast division. *Int J Mol Sci.*, *19*(3), 733.

Yoo, J. H., Cheong, M. S., Park, C. Y., Moon, B. C., Kim, M. C., Kang, Y. H., ... & Cho, M. J. (2004). Regulation of the dual specificity protein phosphatase, DsPTP1, through interactions with calmodulin. *J Biol Chem.*, *279*(2), 848-858.

Yadav, D., Zemach, H., Belausov, E., & Charuvi, D. (2019). Initial proplastid-to-chloroplast differentiation in the developing vegetative shoot apical meristem of Arabidopsis. *Biochem and Biophys Res Commun., 519*(2), 391-395.

Zhang, Z. Y. (2002). Protein tyrosine phosphatases: structure and function, substrate specificity, and inhibitor development. *Annu Rev Pharmacol Toxicol*, *42*(1), 209-234.

Zhang, Y., Li, Q., Wang, Y., & Sun, L. (2018). Biological Function of Calcium-Sensing Receptor (CAS) and Its Coupling Calcium Signaling in Plants.