Characterization of Two MAPK Phosphatases, MKP2 and DsPTP1, and Their Potential MAPK Substrates

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A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirement

for the Degree of Master of Science at

Concordia University

Montreal, Quebec, Canada

September 2023

CONCORDIA UNIVERSITY
School of Graduate Studies

This is to certify that the thesis prepared

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Entitled: Characterization of Two MAPK Phosphatases, MKP2 and DsPTP1, and Their Potential MAPK Substrates

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

Master of Science (Biology)

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ABSTRACT

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Lahouari Zakaria Brahim

In plants and other eukaryotes, mitogen-activated protein kinase (MAPK) cascades are essential signalling pathways that regulate a wide array of cellular functions related to development and environmental stress management responses. MAPK cascades each consist of a chain of kinases ranging from MAPKKKKs to MAPKs, which upregulate downstream kinases by phosphorylation to elicit cellular responses. Ultimately, the cellular output of MAPK signalling is primarily controlled by MAPK phosphatases (MKPs), which downregulate MAPKs by dephosphorylation. The five MKPs identified in *Arabidopsis thaliana* (MKP1, MKP2, DsPTP1, IBR5 and PHS1) have primarily been found to be involved in plant stress responses, making our knowledge of the involvement of these phosphatases in developmental pathways limited. Our research group previously found that MKP2 and DsPTP1 exhibit a striking albino phenotype characterized by small cream-coloured seedlings when both are absent in double mutants. In this thesis, I further characterize the roles of MKP2 and DsPTP1 in plant growth and development and identify potential MAPK substrates of the two phosphatases. *In-silico* transcript and protein accumulation studies were performed and reveal the expression of MKP2 and DsPTP1 in vegetative tissues, indicating their possible role in the early stages of plant development. To identify potential MAPK substrates, yeast two-hybrid and bimolecular fluorescence complementation studies were performed. Based on my analyses, I propose four MAPKs that are potential targets of MKP2/DsPTP1 in a pathway that regulates plant development. In summary, this work characterizes potential roles of MKP2 and DsPTP1 in plant development and identifies potential MAPK substrates for further study.

Acknowledgements

I would like to take this opportunity to express my gratitude and thank everyone who has been instrumental in the successful completion of this master's thesis. Their help and guidance have been invaluable.

First, I would like to express my deepest gratitude to my supervisor, Dr. Jin Suk Lee, whose scholarly mentorship and insightful feedback were instrumental in shaping the present research. Her knowledge, patience and guidance are truly appreciated.

Secondly, I would like to thank committee members Dr. Selvadurai Dayanandan and Dr. Patrick Gulick, and Dr. Madoka Gray-Mitsumune, for their helpful feedback and guidance on this project. I would also like to thank Dr. Vladimir Titorenko for his earlier feedback on the project.

Additionally, I would like to thank fellow lab members Jianlei Sun, Pooja Kaushik, Kritika Bharti, Sehyuk Park and Xutong Wang for the helpful scientific discussions and help in maintaining the lab. Working alongside such enthusiastic and hard-working people has been a great experience.

I would also like to extend my thanks to Concordia University for funding this research and providing the facilities, and to the Centre for Microscopy and Cell Imaging (CMCI) at Concordia University for providing the imaging equipment that made this project possible.

Lastly, I wish to express my gratitude to my parents. Their unwavering support and belief in my abilities are the driving force behind each of my academic endeavours.

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List of Abbreviations

3-AT	3-Amino-1,2,4-triazole
ABA	Abscisic acid
ABI4	Abscisic Acid-Insensitive 4
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
BiFC	Bimolecular fluorescence complementation assay
C	Cysteine
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CDS	Coding sequence
Chl	Chlorophyll
Chl*a*	Chlorophyll *a*
Chl*b*	Chlorophyll *b*
CI	Catalytically inactive
Col-0	Columbia-0 ecotype
D	Aspartic acid
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DSP	Dual-specificity phosphatase
DsPTP1	Dual-specificity Protein Tyrosine Phosphatase 1
E	Glutamic acid
EV	Empty vector
GFP	Green fluorescent protein
IBR5	Indole-3-Butyric Acid Response 5
LB	Luria-Bertani
LHCB	Light-harvesting chlorophyll *a*/*b* binding
M	Methionine
MAPK (or MPK)	Mitogen-activated protein kinase
MAPKK (or MKK)	Mitogen-activated protein kinase kinase

MAPKKK (or MEKK)	Mitogen-activated protein kinase kinase kinase
MKP	Mitogen-activated protein kinase phosphatase
mRNA	Messenger RNA
MS	Murashige and Skoog medium
OX	Overexpression
PAMP	Pathogen-associated molecular pattern
PHS1	Propyzamide-Hypersensitive 1
PTP	Protein tyrosine phosphatase
Q	Glutamine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Serine
SA	Salicylic acid
SAR	Systemic acquired resistance
SD	Synthetic defined minimal medium
T	Threonine
T-DNA	Transfer DNA
TF	Transcription factor
UV	Ultraviolet
V	Valine
WT	Wild type
X	Any given amino acid
Y	Tyrosine
Y2H	Yeast two-hybrid
YFP	Yellow florescent protein

Chapter 1. Introduction

1.1 Mitogen-activated protein kinase (MAPK) cascades

Plants have multiple layers of regulatory mechanisms that allow them to react accordingly to various environmental stimuli. One of the major regulatory pathways found in plants and other eukaryotes is the mitogen-activated protein kinase (MAPK) cascade, a highly conserved signalling pathway. MAPK cascades consist of three main layers of regulation, each of them being controlled by the phosphorylation or dephosphorylation of its respective kinase protein. MAP kinase kinase kinases (MAPKKKs, also known as MEKKs) form the first level of regulation and are at the top of the pathway. Internal and external signals, including environmental stimuli, will generally result in the stimulation of plasma membrane receptors, resulting in the activation of MAPKKKs (Rodriguez et al., 2010). The MAPKKK will, in turn, activate a downstream MAP kinase kinase (MAPKK) by adding two phosphate groups to serine or threonine amino acid residues in a conserved motif (S/T–X_{3-5}–S/T) of the MAPKK activation loop located in the catalytic domain. The addition of phosphate groups to the MAPKK will cause a conformational change in protein structure and will allow for enhanced catalysis, allowing it to phosphorylate its downstream target (Davis, 1993). The activated MAPKK will then continue the phosphorylation sequence by phosphorylating a downstream MAP kinase at threonine or tyrosine amino acid residues of a conserved T–X–Y motif in the activation loop. Activated MAPKs will then activate various effector proteins in the nucleus or cytoplasm to elicit a wide range of cellular responses. Effector proteins include transcription factors, enzymes, and other downstream kinases which serve different biological functions. Additionally, there is also a fourth layer of regulation present as the MAP kinase kinase kinase kinases (MAPKKKKs). Although their regulatory functions are poorly understood, MAPKKKKs might be involved in linking the upstream steps of the cascade to the pathway beginning with the activation of MAPKKKs (Colcombet & Hirt, 2008).

1.2 MAPKs

Mitogen-activated protein kinases (MAPKs) are dual-specificity kinases involved in MAPK signalling. They interact specifically with the serine and threonine amino acids of their target substrates to trigger precise responses in the cell as a result of the MAPK cascade (Marshall, 1994). The roles of MAPKs are vast and include various biological processes such as cell cycle

regulation, plant growth and development, hormonal and stress responses, and innate immunity. (Krysan & Colcombet, 2018).

So far, 80 MAPKKKs, 10 MAPKKs, and 20 MAPKs encoded by MAPK cascade-specific genes have been discovered in *Arabidopsis thaliana* (Colcombet & Hirt, 2008). The 20 Arabidopsis MAPKs all contain the conserved T–X–Y motif, which is the phosphorylation target of upstream MAPKKs, and are further divided into four different groups. Groups A, B, and C are characterized by a common T–E–Y motif at their phosphorylation sites and are known as TEY subtype MAPKs. Group A MAPKs are unique in that they have a T–Q–Y motif in addition to the T–E–Y motif. Group B MAPKs have other motifs in addition to the previous two: T–E–Y, T–E–C, and T–V–Y, which are unique to three of its members. Three of the MAPK members of group C also have unique motifs, including T–S–Y and M–S–Y. Group D is evolutionarily divergent from the other groups and is characterized by a T–D–Y motif at the phosphorylation site (Bigeard & Hirt, 2008). MAPKs part of group D are known as TDY subtype MAPKs and do not feature unique MAPK motifs (other than T–D–Y), unlike MAPKs belonging to the other three groups. Many of the group A, B, and C Arabidopsis MAPKs have similarly named homologues in other plant species, such as spruce, rice, maize, cucumber, and tobacco (Jagodzik et al., 2018). The MAPK members of each group are MPK3/6/10 (group A), MPK4/5/11–13 (group B), MPK1/2/7/14 (group C), and MPK8/9/15–20 (group D). The group E and F subfamilies of MAPKs are not currently known to include any Arabidopsis MAPKs but include MAPKs from species such as *Chlamydomonas reinhardtii* and *Volvox carteri* (Jagodzik et al., 2018).

MPK3 and MPK6 are the most well-studied MAPKs from group A. MPK3 and MPK6 are known to be negative regulators of stomatal development and patterning, a process where they are positively regulated by upstream kinases, MKK4/5 (Wang et al., 2007). The MKK4/5–MPK3/6 module is also known to be located downstream of YODA, a MAPKKK which represents another layer of regulation (Bergmann et al., 2004; Wang et al., 2007). The most studied functions of MPK3 and MPK6 are related to plant stress responses, namely the oxidative stress response and immunity. Along with MKP2, a MAPK phosphatase, MPK3 and MPK6 control oxidative stress tolerance; they are transiently activated during exposure to reactive oxygen species (ROS) and are negatively regulated by MKP2, attenuating sensitivity to oxidative stress (Lee & Ellis, 2007). Additionally, MPK3 and MPK6 negatively regulate innate immunity in response to pathogens (Lumbreras et al., 2010). MPK3 and MPK6 also have plant

development-related functions; a loss of function of MPK3/6 results in clustered stomata, indicating their function in coordinated cell fate specification. Other important functions of MPK3/6 include embryogenesis, where their loss of function results in embryo lethality (Xu et al., 2014), and various biotic and abiotic stress-related functions (Beckers et al., 2009).

The group B subfamily of MAPKs is relatively less studied than its group A counterpart. MPK4, a member of group B, is involved in plant cytokinesis during meiosis and mitosis (Kosetsu et al., 2010) and also has regulatory functions in PAMP-triggered immunity (Berriri et al., 2012; Bazin et al., 2020). In Arabidopsis, the *mpk4* mutation results in a higher accumulation of salicylic acid (SA), which in turn results in higher expression levels of pathogenesis-related (PR) genes and therefore enhanced resistance to various pathogens (Petersen et al., 2000; Lin & Chen, 2018). MPK4 is, therefore, a negative regulator of SA levels and systemic acquired resistance (SAR), which is dependent on elevated salicylic acid levels. In terms of plant development, the *mpk4* loss of function also results in immature cell plates and dwarf phenotypes, which indicates that MPK4 is a positive regulator of plant cytokinesis and is required for proper meiotic and somatic cytokinesis (Kosetsu et al., 2010). Plant cytokinesis also involves MKP11 as increased MKP11 transcript levels are detected in *mpk4* plants (Kosetsu et al., 2010). Physical interaction studies show that MEK1 and MKK2 are two upstream MAPKKs that physically interact with MPK4 (Ichimura et al., 1998). Protein kinase assays also reveal that MEK1 and MKK2 have the ability to phosphorylate and activate MPK4, both *in vitro* and *in vivo* in the case of MKK2 (Huang et al., 2000; Teigen et al., 2004). MEK1 is currently only known to phosphorylate MPK4 *in vitro* (Huang et al., 2000).

Group C MAPKs are the least studied MAPKs in Arabidopsis, and little is known about their biological functions. Phosphoproteomic studies, however, reveal that the Group C MAPKs (MPK1/2/7/14) are each activated by abscisic acid (ABA), a phytohormone involved in multiple plant development and stress-related processes, and form a regulatory module located downstream of MAPKKK17/18 and MKK3, which directly regulate them as part of the cascade (Danquah et al., 2015).

Compared to the members of the group A and B subfamilies of MAPKs, the biological functions of group D MAPKs are relatively poorly understood, being evolutionarily divergent from the rest. MPK8 is known to be involved in seed germination in conjunction with TCP14, a seed germination-related transcription factor (Zhang et al., 2019). Seeds that present an *mpk8* loss of

function exhibit a deeper dormancy than wild-type seeds, indicating the role of MPK8 as a positive regulator of seed germination. MPK15 is involved in PAMP-triggered immunity, where its phosphorylation grants the plant powdery mildew fungal resistance (Shi et al., 2022). It is also known that the upstream receptor that allows the signal transduction eventually resulting in the phosphorylation of MPK15 is BSK1, but it is unknown which MAPKK is responsible for phosphorylating and activating MPK15.

All in all, MAPKs have been demonstrated to have a diverse array of regulatory functions in multiple plant processes, including cell proliferation, cell differentiation, immunity, and responses to abiotic stresses, where they act in the transduction of extracellular signals into cellular responses as part of MAPK cascades.

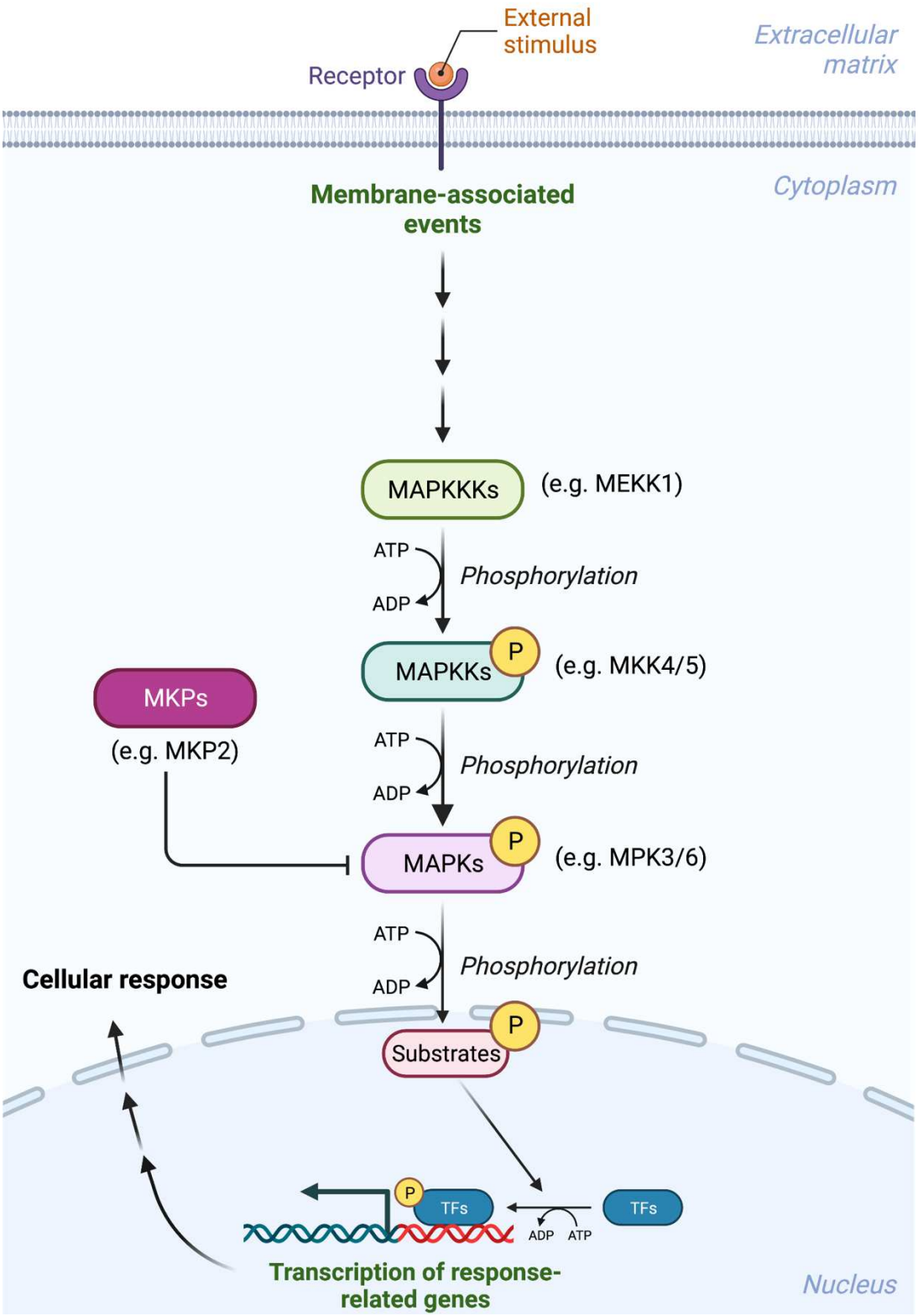


Figure 1. Review diagram of a typical MAPK cascade in eukaryotes. MAPK cascades are comprised of regulatory kinase modules that sequentially act on a downstream target. MKPs negatively regulate the cascade by having a dephosphorylating effect on MAPKs. The diagram was created in BioRender.

1.3 Mitogen-activated protein kinase phosphatases (MKPs)

While the different MAPK components of MAPK cascades are activated and regulated by each MAPK's respective upstream kinase, the mechanism of negative regulation, dephosphorylation/deactivation of the kinases, relies on the inhibitory action of MAP kinase phosphatases (MKPs), which remove added phosphate groups from activated MAPKs in order to deactivate them. MKPs are dual-specificity phosphatases; they function by removing the phosphate groups from both the threonine and tyrosine residues of activated MAPKs, which are normally added by MAPKKs as part of the MAPK cascade (Liu et al., 2007). The balance between the phosphorylating/activating effect of upstream MAPKKs and the dephosphorylating/deactivating effect of MKPs allows for the fine-tuning of the cell's physiological responses to environmental stimuli.

MKPs, which act directly on MAPKs, are important regulators of multiple plant processes owing to their relationship with MAPKs. They have been shown to be involved in the innate immune response, oxidative stress regulation, osmotic stress regulation, and ABA signalling (Lee & Ellis, 2007; Lumbreras et al., 2010; Liu et al., 2015). The roles of MAPKs are known to be diverse and include a wide range of biological functions, including essential roles in plant development, but so far, most studies describing the roles of MKPs are mainly limited to roles related to stress responses (e.g., oxidative, osmotic, genotoxic and anti-pathogen stress responses). Considering that MAPK activity is tightly balanced by the activating action of upstream MAPKKs, as well as negative regulators, MKPs, and that MAPK signalling is crucial in controlling many aspects of plant growth and development, it is hypothesized that MKPs might have novel roles in plant development owing to their relationship with MAPKs.

While 23 dual-specificity (Ser/Thr and Tyr) phosphatases (DSPs) have been identified in the *Arabidopsis thaliana* genome, five of them are considered functional MKPs, owing to their unique AY[L/I]M motif that allows them to interact with and dephosphorylate MAPKs in a dually specific manner (Kerk et al., 2002, 2008). MAPKs consist of two domains: the non-catalytic N-terminal domain, which is the site of MAPK binding and is responsible for substrate specificity, and the C-terminal domain, which is the site of catalysis where an arginine residue interacts with phosphotyrosine or phosphothreonine for the purpose of dephosphorylation (Farooq et al., 2001). The five MKPs present in *Arabidopsis thaliana*, as well as other plant species as homologues, are Mitogen-Activated Protein Kinase Phosphatase 1 (MKP1), Mitogen-

Activated Protein Kinase Phosphatase 2 (MKP2), Dual-specificity Protein Tyrosine Phosphatase 1 (DsPTP1), Indole-3-Butyric Acid Response 5 (IBR5), and Propyzamide-Hypersensitive 1 (PHS1).

DsPTP1 was the first MKP that was shown to be capable of dephosphorylating and inactivating a downstream MAPK, MPK4, *in vitro* (Gupta et al., 1998). It is a dual-specificity MAPK phosphatase and specifically catalyzes the cleavage of phosphate groups on phosphotyrosine and phosphoserine/phosphothreonine amino acid residues. DsPTP1 is known to be a negative regulator of osmotic stress signalling during seed germination (Liu et al., 2015). Its other known function is as a positive regulator of ABA accumulation when under osmotic stress (Liu et al., 2015).

MKP1 is involved in multiple stress responses, which are regulated through its interaction with MPK3, MPK4, and MPK6 both *in vitro* and *in vivo* (Ulm et al., 2002; Bartels et al., 2009). It is known as a positive regulator of the genotoxic stress response as *mkp1* loss-of-function Arabidopsis plants show hypersensitive phenotype when exposed to genotoxic stresses (exposure to MMS and UV-C radiation) (Ulm et al., 2001). Additionally, MKP1 also has a significant negative regulatory role in salt stress tolerance. Loss-of-function *mkp1* mutants show higher resistance to plant stress at the vegetative stage when compared to wild-type controls (Ulm et al., 2002). More recently, MKP1 was found to have a novel role in stomatal development, where it controls stomatal cell fate transition at the early stages of development by having an inhibiting effect on MPK3 and MPK6 (Tamnanloo et al., 2018). This finding represents a novel case of an Arabidopsis MKP being found to be involved in plant development and contrasts with previous Arabidopsis MKP research, which primarily focused on the environmental stress regulation and immunity aspects of MKP function.

MKP2 is a dual-specificity MAPK phosphatase; it catalyzes the cleavage of phosphate groups on both the serine and threonine residues in the activation loop of its downstream substrates (Camps et al., 2000). The first role that was found for MKP2 is its role in the oxidative stress response. It positively regulates the response to oxidative stress by dephosphorylating and deactivating MPK3 and MPK6 in Arabidopsis (Lee & Ellis, 2007). Additionally, MKP2 is also a positive regulator of the innate immune response in plants, in which it functions by dephosphorylating and deactivating MPK3 and MPK6. Plants exhibiting an MKP2 loss of function showed delayed

wilting symptoms and accelerated disease progression after pathogen infection (Lumbreras et al., 2010).

IBR5 is a regulator of the auxin and abscisic acid (ABA)-mediated signalling pathways and interacts with MPK12 *in vitro* and *in vivo* (Monroe-Augustus et al., 2003; Lee et al., 2009). Arabidopsis plants that exhibit the *ibr5* mutation show attenuated phenotypes in response to ABA and auxin compared to the wild-type controls, where root elongation and germination are more severely inhibited (Monroe-Augustus et al., 2003). IBR5 is also confirmed to interact with MPK12, whose loss-of-function mutation complements the phenotype observed in *ibr5* plants (Lee et al., 2009).

PHS1 is known to be involved in ABA signalling and physically interacts with MPK12 and MPK18. Loss-of-function *phs1-1* mutants exhibit inhibited root elongation, and *phs1-3* plants show suppression of seed germination when exposed to applied exogenous ABA (Quettier et al., 2006). The other known function of PHS1 is in microtubule polymerization (Naoi & Hashimoto, 2004; Pytela et al., 2010). Additionally, PHS1 is a positive regulator of flowering, which it controls by regulating the expression of two flowering-related transcription factors, CO and FLC (Tang et al., 2016).

Although the five Arabidopsis MKPs have been linked to different stress responses in *Arabidopsis thaliana*, their roles remain relatively poorly understood when considering the broader range of biological functions associated with MAPKs, which are directly negatively regulated by MKPs.

1.4 Interactions of Arabidopsis MAPKs with MKPs

So far, out of the five MKPs present in Arabidopsis, MKP1, MKP2, IBR5, and PHS1 have known downstream MAPK interactors. The MAPK substrates for each MKP can vary depending on the biological context, and interactions are generally established for the regulation of specific biological processes, many of which have yet to be elucidated.

MKP1 is known to interact with MPK3, MPK4, and MPK6 as part of its roles in salt and UV stress tolerance, immunity, and plant development. *In-vivo* transient assays reveal that MKP1 inactivates MPK3/6 when co-expressed in Arabidopsis protoplasts and results in lower protein expression, indicating a potential phosphatase–kinase substrate relationship (Asai et al., 2002). Other assays, such as yeast two-hybrid (Y2H) screens, have also revealed that MKP1 physically

interacts with MPK3/4/6, with it showing a stronger interaction with MPK6 and equal, medium-strength interactions with MPK3 and MPK4 (Ulm et al., 2002). In addition to its abiotic and biotic stress-related functions, MKP1 has been demonstrated to functionally interact with MPK3 and MPK6 as part of its role in regulating stomatal development (Tamnanloo et al., 2018).

MKP2 is known to negatively regulate MPK3 and MPK6 as part of the oxidative stress and innate immune responses, where it dephosphorylates the two MAPKs to positively regulate either of these two biological responses. Moreover, dephosphorylation assays reveal that MKP2 dephosphorylates both phospho-MPK3 and phospho-MPK6 *in vitro*, demonstrating that the MKP2–MPK3/6 interaction represents an enzyme–substrate relationship (Lee & Ellis, 2007). *In planta*, bimolecular complementation studies, where MKP2 is shown to physically interact with MPK3 and MPK6 when co-infiltrated in tobacco leaves, also corroborate this finding (Lumbreras et al., 2010).

IBR5 is currently known to only have MPK12 as an interactor *in vivo* and *in vitro*. Yeast two-hybrid screens and co-immunoprecipitation assays show that MPK12 physically interacts with IBR5 (Lee et al., 2009). The interaction is also found *in planta* when IBR5 and MPK12 are expressed in Arabidopsis protoplasts. Furthermore, *in vivo* phosphatase assays indicate that phospho-MKP12 is efficiently dephosphorylated by IBR5 (Lee et al., 2009), indicating an enzyme–substrate relationship.

PHS1 has previously been found to physically interact with MPK12 and MPK18 in yeast two-hybrid screens, but no physiological role has been elucidated for the MPK12 interaction. PHS1 is involved in microtubule polymerization, where it functionally interacts with MPK18 as a substrate, and ABA signalling (Naoi & Hashimoto, 2004; Quettier et al., 2006). *In planta*, BiFC assays where the proteins are co-infiltrated in tobacco epidermal cells also corroborate the physical PHS1–MPK18 interaction (Walia et al., 2009).

Although DsPTP1 was previously found to be capable of dephosphorylating the recombinant MPK4 protein *in vitro* (Gupta et al., 1998), there is no evidence of physical interaction between them. This possible enzyme–substrate relationship has also not been associated with either of the two elucidated roles of DsPTP1 in Arabidopsis stress responses (ABA accumulation and osmotic stress management during seed germination). None of the Arabidopsis MAPK have yet been identified as DsPTP1 substrates *in planta* and *in vivo* or have been associated with the currently

known functions of DsPTP1, which suggests that some of the biological functions of DsPTP1 have yet to be elucidated.

Despite most described MAPKs having various roles in plant development-related functions and being directly negatively regulated by MKPs, it can be observed that Arabidopsis MKPs (apart from MKP1, which is involved in stomatal development) have mainly been studied to elucidate their roles in abiotic stress responses and immunity. The close enzyme–substrate relationship between MKPs and MAPKs in MAPK signalling pathways indicates that MKPs are highly likely to be involved in the same processes as MAPKs. This leaves many plant development processes in which MKPs might be involved that have yet to be studied.

1.5 MAPK signalling in chloroplast biogenesis

While MAPK cascades are known to be involved in stress responses and immunity in plants, their precise roles in chloroplast development remain relatively poorly understood. So far, it is known that at least one MAPKKK is involved in chloroplast development and that its absence can cause seedling-lethal phenotypes. In rice, OsCSL1 encodes MAPKKK12, and its absence results in chloroplast-related defects like yellowed leaves, and death at the trefoil stage. The WT phenotype is fully restored by functional complementation (Liang et al., 2022). Additionally, it was found that MKKK22 interacts with downstream MKK4 as part of the pathway (Liang et al., 2022). In Arabidopsis, at least two MAPKs, MPK3 and MPK6, are known to be involved in retrograde signalling (i.e., signalling from plastids to the nucleus) during chloroplast biogenesis. MPK3 and MPK6, which are initially activated by two upstream MAPKKs, MKK4 and MKK5, were found to phosphorylate and activate ABI4, a transcription factor that negatively regulates the transcription of photosynthetic genes (including the *LHCB* gene family) by binding specific promoter sequences (Guo et al., 2016), which in turn contributes to fine-tuning chloroplast function and development. Guo et al.'s (2016) model also states that the MKK4/5–MPK3/6 module binds 14-3-3ω, a Ca^{2+}-dependent scaffolding protein whose role is to transport MPK3/6 to the nucleus, where it can activate ABI4 to repress the transcription of various photosynthetic genes, most notably the six *LHCB* (*Light-harvesting chlorophyll a/b binding*) genes, which are collectively responsible for encoding the apoproteins of the light-harvesting complex of photosystem II (Teramoto et al., 2001; Liu et al., 2013). MKK4-5–MPK3/6 binding to 14-3-3ω is further positively regulated by CAS-mediated Ca^{2+} signalling, another layer of regulation that

ensures that the scaffolding process only occurs when sufficient Ca^{2+} levels are available in the cytosol (Guo et al., 2016).

Chloroplasts are photosynthetic organelles found in plants and photosynthetic algae that evolved from ancestral cyanobacterial symbionts 1.5 billion years ago (Pogson et al., 2011, 2015). Each chloroplast forms a photosynthetic compartment that is comprised of a double-layered membrane on the outside and stacks of membranous discs called thylakoids (each stack being a granum) surrounded by stroma on the inside. While chloroplasts are also involved in nitrogen and sulphur assimilation, and the synthesis of some amino acids and hormones, one of the most important roles of the chloroplast compartment is its role in photosynthesis. Chloroplasts house most of the photosynthetic machinery and contain the main sites of photosynthesis: the thylakoid membranes, which is where the electron transport chain is established and the light-dependent reactions occur, and the stroma, which is the site where the Calvin-Benson-Bassham converts the energy molecules produced by photosynthesis and uses them to synthesize hexose sugars, which can be used by plants as a final energy source (Raines, 2003).

Photosynthesis uses a set of photosynthetic protein complexes located in the thylakoids to generate NADPH and ATP from sunlight and environmental carbon dioxide. The generated NADPH and ATP are initial energy sources that can later be converted into sugars. In the stroma, the Calvin-Benson-Bassam cycle (also known as the “dark” cycle) will then use NADPH and ATP to generate G-3-P, a precursor molecule to the sugars the plant needs. The resulting G-3-P is then used to synthesize hexose sugars.

In dicots, chloroplast development consists of different stages that vary depending on the specific type of chloroplast being differentiated. In Arabidopsis, the protoplastids are the initial precursor organelles to chloroplasts. Protoplastids are hypothesized to first develop into an etioplastid intermediate form, which contains a prolamellar body (PLB) where prothylakoids start developing and differentiating into thylakoids. The newly formed chloroplasts then develop into mature chloroplasts that are capable of photosynthesis.

In photosynthetic organisms, albinism is most often indicative of a severely reduced level of chlorophyll pigments in normally green tissues and is associated with the inability of protoplastids to develop into mature chloroplasts properly (Silva et al., 2020). It is generally the result of a genetic mutation that hinders chloroplast development and affects multiple aspects of plant development.

1.6 Thesis objectives

Although MKPs make up the first level of regulation of MAP kinase activity along with MAPKKs, which are known to be involved in a wide range of development-related functions, few development-related functions of MKP2 or DsPTP1 have been elucidated thus far. The significance of the project lies in the possibility that MKP2 and DsPTP1, neither of which is currently known to be involved in plant development-related functions (other than seed germination under osmotic stress), might be involved in development-related processes in conjunction with downstream MAPK substrates.

Based on preliminary evidence of an albino lethal, chloroplast biogenesis-related phenotype in *mkp2 dsptp1* double mutants, our research group attempted to further characterize the roles of MKP2 and DsPTP1 in plant development and their potential MAPK substrates.

Preliminary assays were therefore performed and used to establish candidate MAPKs that might functionally interact with MKP2 and DsPTP1 (Lee Lab, unpublished work). This allowed us to focus on MPK8 and MPK15, two MAPKs that showed signs of positive interaction in preliminary assays. As per the literature, the known interactors of MKP2 (MPK3 and MPK6) and the potential substrate of DsPTP1 (MPK4), along with MPK8 and MPK15, were considered candidate MAPK substrates of MKP2 and DsPTP1 and were thereby studied.

In light of the preliminary findings, the primary objectives of the study are twofold: firstly, to gain insight into the function of two MKPs, MKP2 and DsPTP1, regarding their involvement in plant development, and, secondly, to identify potential MAPK substrates associated with each of the two phosphatases.

The first aim is explored through *in-silico* expression analyses and protein accumulation studies at different stages of plant development. Other factors like the photosynthetic pigment content and the flower and root phenotypes of transgenic lines are investigated to further characterize the phenotypes associated with MKP2 and DsPTP1 function. The study also aims to investigate interactions of MKP2 and DsPTP1 with potential MAPK substrates (MPK3/4/6/8/15) through physical interaction studies. These investigations will ultimately contribute to the identification of a novel MAPK signalling pathway controlling plant growth and development.

Chapter 2. Materials and Methods

2.1 *In-silico* analysis of *MKP2* and *DsPTP1* tissue-specific expression patterns

The Arabidopsis eFP Browser 2.0, developed by Winter et al. (2007), was used to obtain visual outputs of relative and absolute tissue-specific gene expression patterns in *Arabidopsis thaliana*. The outputs were used to plot graphs showing the relative tissue-specific expression patterns and expression intensities of the *MKP2* and *DsPTP1* transcripts in comparison to a reference gene. The online tool is accessible on the BAR web server at http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFPBrowser2.html.

2.2 Plant materials and plant growth conditions

The *Arabidopsis thaliana mkp2-2* (SALK_045800) and *dsptp1-1* (SALK_092811) single mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). Wild-type (WT), and *mkp2-2/+ dsptp1-1* and *mkp2-2 dsptp1-1/+* seeds were used for generating the transgenic plants in WT and double mutant backgrounds, respectively. All the Arabidopsis lines used in this study are of the Columbia-0 (Col-0) ecotype. The *mkp2-2/+ dsptp1-1* and *mkp2-2 dsptp1-1/+* higher-order mutant seeds were obtained by crossing the mutants and were propagated in a state of heterozygosity due to the seedling-lethal effect of the homozygous double mutation.

For sterilization, Arabidopsis seeds are initially aliquoted in microcentrifuge tubes to be sterilized by incubating with shaking for 9-10 minutes in a sterilization solution (33% bleach, 0.1% Triton X-100, ddH_2O). The seeds are then washed five times with sterile ddH_2O to get rid of any remaining bleach. Sterile 0.01% agar is then added to the sterile seed vial to facilitate sowing on half-strength Murashige and Skoog (½ MS, pH 5.7) medium plates using pipette tips. The plates are then left at 4 °C in the dark for 3 days to ensure the even growth of every seedling. Following the cold treatment, the ½ MS plates containing the seeds are moved to an E15 Conviron growth chamber at a light intensity of 100-120 μmol m^{-2} s^{-1} and a temperature of 22 °C under 16-hour light/8-hour dark cycle conditions.

For plants that are observed at later stages of development, the seedlings are transplanted from ½ strength MS plates to soil pots (mixture of 2 parts black earth, 1 part peat moss, and 1 part vermiculite) at 10 days post-germination (dpg). The transplanted plants are then grown in an E15 Conviron growth chamber at the conditions described above.

2.3 Plasmid construction

Our research group utilized the Gateway cloning system (Invitrogen) to generate the plasmids used in this study. A detailed description of the plasmids can be found in Table 1.

2.4 Agrobacterium-mediated transformation

The floral-dip method for *Agrobacterium tumefaciens* (Agrobacterium)-mediated transformation was used to generate the transgenic plant lines used in this study (Clough & Bent, 1998; Zhang et al., 2006). A seed culture of LB medium with the appropriate antibiotics is inoculated with *Agrobacterium tumefaciens* cells of the GV3101 strain containing the transgenic plasmid construct and grown overnight (for 12-16 hours) at 28 °C with shaking at 200 rpm. Four hundred microlitres of grown seed culture were then used to inoculate 400 mL of LB medium with the appropriate antibiotics for selection. The resulting culture is grown at 28 °C with shaking at 200 rpm for 24 more hours. After incubation, the culture is poured into bottles (Nalgene Oak Ridge) and pelleted using a Beckman Coulter Avanti ultracentrifuge at 6000 rpm set to 4 °C for 17-20 minutes.

The resulting pellets are then resuspended in the floral dip solution (5% sucrose, 0.05% Silwet L-7, sterile ddH_2O). When the main stems of plants reach a length of 10 to 15 cm, they are dipped in the Agrobacterium dipping solution for 3 seconds, laid on their side, and fully covered with an opaque plastic tray to create a dark environment. The plants are then kept in the dark overnight (for 12-16 hours). The next day, plants are put back in the upright orientation and watered. The floral dipping process is repeated one week later to ensure the high transformation efficiency of T_0 plants. The putative transformant seeds are then screened on antibiotic plates and grown and propagated to the T_3 generation, with antibiotic screening being repeated for each generation. Plants were selected for single T-DNA insertions and homozygosity.

2.5 DNA extraction and PCR genotyping

A small piece of leaf tissue is initially sampled from plants older than 3 weeks using scissors. The leaf sample is then submerged in DNA extraction buffer (0.5% SDS, 250 mM NaCl, 200mM Tris-HCl pH7.5, 25 mM EDTA, and sterile ddH_2O) in a microcentrifuge tube and ground using a small pestle. If multiple samples are being ground, they are kept in ice during this step. After being ground, the samples are centrifuged at 13,500 rpm at 4 °C for 7 minutes to separate the

debris from the extracted DNA in the solution. One hundred fifty microlitres of supernatant are then transferred to a new microcentrifuge tube, where 150 µL of isopropanol is added. The tube is then mixed by inverting and is left at room temperature for 2 minutes. It is then centrifuged at 13,500 rpm for 10 minutes, and the supernatant is discarded. The pellet is then washed with 500 µL of 70% ethanol and is centrifuged at 13,500 rpm for 5 minutes. The resulting supernatant is then discarded, and the pellet is dried using a speed vacuum for 20 minutes. Following this, the DNA pellet is resuspended in 40 µL of sterile ddH_2O for PCR genotyping.

PCR genotyping was done by adding gene-specific primers to a mixture of extracted DNA (1-2 µL) and master mix (5 U Taq DNA polymerase, 200 µM dNTPs, 1X reaction buffer, 2 mM $MgSO_4$ and 5.45 µL sterile ddH_2O per reaction). PCR was then performed on the DNA reaction mix for 32-35 cycles using a Bio-Rad T100^{TM} thermal cycler. Following this, gel electrophoresis using a 1% agarose gel was performed on the samples to identify gene amplification at specific loci.

Mutant genotypes were verified by PCR-based analysis using primers *mkp2-2* LP (5'-TGTCTTAACCGTTGCTGTGG-3'), *mkp2-2* RP (5'-CTGGTTTGGGTATGGGATTG-3'), *dsptp1-1* f (5'-TCCCTTCCCTTATTGAACAGG-3'), *dsptp1-1* rc (5'-AAACAATGACAGCCCATGAAC-3'), and LBa1 (5'-TGGTTCACGTAGTGGGCCATCG-3').

2.6 Microscopy

For the cellular protein localization assays, seedlings were observed at 3 and 8 days post-germination using a C2/TIRF confocal microscope set to show GFP and DIC channels at 20X with the "zoom 2" setting. For each line, representative whole seedlings were imaged at 3 dpg. At 8 dpg, the leaf epidermis, root elongation zone, and root tip were specifically imaged to assess protein localization. At 4 weeks, parts of interest were observed for each line using the confocal microscope.

For the seed segregation analysis, transgenic and wild-type Arabidopsis plants approaching maturity (3 to 4 weeks old) were observed under a stereo microscope. The siliques were first cut from the plant and then cut laterally along the length of the silique using a surgical blade. While slicing the silique open, the pedicel is held with fine forceps (Dumont-Inox #4) with the non-dominant hand to support it. The open siliques were viewed using a Leica stereo microscope

with a camera attachment using incidental lighting at a total magnification of 20X. A ruler was used to determine the scale.

2.7 Photosynthetic pigment quantification assay

One hundred milligrams of seedlings were ground on ice using pestles and liquid nitrogen. The ground tissues were then suspended in 400 µL 80% (v/v) acetone and centrifuged at 13,250 RPM at 4 °C for 10 minutes. Following this, cycles of suspension/centrifugation in 200 µL 80% acetone were repeated multiple times, and the extract was retained and pooled with previous extracts until each pellet became white in colour, indicating that the chlorophyll was fully dissolved in the solution. For each sample, the absorbances of 1/10 diluted samples were measured at 663 nm, 646 nm, and 470 nm, with the spectrophotometer being blanked at 750 nm. Five technical replicates were taken for each batch of seedlings. The Lichtenthaler & Wellburn (1983) equations for 80% acetone were then used to quantify the levels of chlorophyll *a*, chlorophyll *b,* and carotenoid pigments.

$C_a = 12.21\ A_{663} - 2.81\ A_{646}$

$C_b = 20.13\ A_{646} - 5.03\ A_{663}$

$C_{carotenoid} = (1000\ A_{470} - 3.27\ C_a - 104\ C_b) / 229$

2.8 Phenotypic assays

For phenotypic assays, seeds were sown on ½ MS plates, and seedlings were grown as previously described. Phenotypic assays were conducted at 3, 8, and 5 weeks post-germination. Images of seedlings and mature plant parts (inflorescences, flowers and siliques) were captured using a phone camera attached to a Nikon dissecting microscope.

For root length assays, seeds were grown for 10 days on ½ MS plates placed vertically to allow roots to grow parallel to the medium. Roots were then imaged using a phone camera and measured using the measurement function in ImageJ. A ruler was used to set the scale.

2.9 Bimolecular fluorescence complementation and transient expression assays

Four-week-old *Nicotiana benthamiana* plants were agroinfiltrated with cultures of transgenic *Agrobacterium tumefaciens* (strain GV3101) to visualize protein interactions *in vivo*. Cultures of

10 mL of YEB medium inoculated with transgenic Agrobacterium containing binary plasmids (fusion constructs with either nYFP or cYFP) were grown overnight (12-16 hours) at 30 °C with shaking at 200 rpm. Each culture was then centrifuged for 5 minutes at 5,000 rpm, and the pellets were resuspended in an infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 150 μM acetosyringone, pH = 5.6). The resulting cultures were then incubated at RT for 4-6 hours before agroinfiltration. For each tobacco plant, the abaxial (lower) sides of three leaves were infiltrated using 1 mL syringes loaded with the *A. tumefaciens* in the infiltration buffer. The leaves were infiltrated until at least 80% of each leaf was visibly covered with solution. The leaves were infiltrated with one or two different *A. tumefaciens* cultures, depending on the interactions to test. Following agroinfiltration, the plants were incubated at growth chamber conditions for 48-60 hours (before protein aggregation could occur), after which imaging was performed.

For visualizing the interactions, the Nikon C2/TIRF confocal microscope was used. The microscope was set to detect the appropriate wavelength for yellow fluorescent protein (YFP) signals. Slides were prepared using small cut pieces of leaf epidermis laid as flat as possible. One drop of 15% glycerol was then added to the abaxial side of the leaf epidermis sample, which was then covered by a coverslip. Type FF immersion oil was used to view the samples with the 40X objective lens at a zoom setting of 1.

For Y2H screening, YPDA medium was inoculated with a transgenic yeast stock (strain AH109) containing fusion plasmids with either the AD or BD domain and grown overnight (12-16 hours) at 28 °C with shaking at 200 rpm. The cultures were then re-cultured for 3-4 hours until the OD_{600} reached a value of 1 (using 1 mL of culture for measurement). Then, the culture was centrifuged to form a pellet, which was washed twice with TE buffer and resuspended in 0.1 M LiOAc. Thirty microlitres of salmon sperm DNA (1 mg/mL) and 350 μL 50% (w/v) PEG3350 were added to the suspension. Then, 1-2 μL of plasmid DNA was added (for a concentration of approximately 500 ng/mL plasmid DNA), and the culture was kept at RT for one hour. Following this, 250 μL of the mixture is spread onto minimal SD/-Leu/-Trp/-His (selective medium) and SD/-Leu/-Trp (transformation control) plates.

To confirm the results, colonies were picked, suspended in sterile water, and re-spread onto selective plates containing varying concentrations of 3-Amino-1,2,4-triazole (3-AT) (0-10 mM). The colonies were then sampled and stamped onto selective and non-selective plates and grown for 3 days.

2.10 Tables

Table 1. List of plasmids used in the study.

Description	Insert	Vector	Purpose
MKP2pro::MKP2	*proMKP2* + *MKP2* cDNA (no stop)	pR4 501	Plant transformation
DsPTP1pro::DsPTP1	*proDsPTP1* + *DsPTP1* cDNA (no stop)	pR4 501	Plant transformation
pro35S::GFP	-	pGWB5	Plant transformation
pro35S::MKP2-GFP	*MKP2* cDNA (no stop)	pGWB5	Plant transformation
pro35S::DsPTP1-GFP	*DsPTP1* cDNA (no stop)	pGWB5	Plant transformation
pro35S::DsPTP1-4xMyc	*DsPTP1* cDNA (no stop)	pGWB17	Plant transformation
pBaTL-nYFP (without ccdB)	-	pBaTL-nYFP	BiFC
MKP2-nYFP	*MKP2* cDNA (no stop)	pBaTL-nYFP	BiFC
CIMKP2-nYFP	*CIMKP2* cDNA (no stop)	pBaTL-nYFP	BiFC
DsPTP1-nYFP	*DsPTP1* cDNA (no stop)	pBaTL-nYFP	BiFC
CIDsPTP1-nYFP	*CIDsPTP1* cDNA (no stop)	pBaTL-nYFP	BiFC
pBaTL-cYFP (without ccdB)	-	pBaTL-cYFP	BiFC
DsPTP1-cYFP	*DsPTP1* cDNA (no stop)	pBaTL-cYFP	BiFC
MPK3-cYFP	*MPK3* cDNA (no stop)	pBaTL-cYFP	BiFC
MPK4-cYFP	*MPK4* cDNA (no stop)	pBaTL-cYFP	BiFC
MPK6-cYFP	*MPK6* cDNA (no stop)	pBaTL-cYFP	BiFC
MPK8-cYFP	*MPK8* cDNA (no stop)	pBaTL-cYFP	BiFC
MPK15-cYFP	*MPK15* cDNA (no stop)	pBaTL-cYFP	BiFC
MKP2 in PGBKT7	*MKP2* cDNA (no stop)	pGBKT7	Y2H
DsPTP1 in pGBKT7	*DsPTP1* cDNA (no stop)	pGBKT7	Y2H
MPK3 in pGADT7	*MPK3* cDNA (no stop)	pGADT7	Y2H
MPK4 in pGADT7	*MPK4* cDNA (no stop)	pGADT7	Y2H
MPK6 in pGADT7	*MPK6* cDNA (no stop)	pGADT7	Y2H
MPK8 in pGADT7	*MPK8* cDNA (no stop)	pGADT7	Y2H
MPK15 in pGADT7	*MPK15* cDNA (no stop)	pGADT7	Y2H

Table 2. List of transgenic plants used for the study.

Transgenic plant	Background	Plasmid	Vector	Purpose
proMKP2::MKP2 in *mkp2-2 dsptp1-1*	Double mutant	*MKP2pro::MKP2*	pR4 501	Seed analysis
proDsPTP1::DsPTP1 in *mkp2-2 dsptp1-1*	Double mutant	*DsPTP1pro::DsPTP1*	pR4 501	Seed analysis
pro35S::GFP	Wild type	*pro35S::GFP*	pGWB5	Protein localization / Overexpression studies
pro35S::MKP2-GFP	Wild type	*pro35S::MKP2-GFP*	pGWB5	Protein localization / Overexpression studies
pro35S::DsPTP1-GFP	Wild type	*pro35S::DsPTP1-GFP*	pGWB5	Protein localization / Overexpression studies
pro35S::DsPTP1-4xMyc	Wild type	*pro35S::DsPTP1-4xMyc*	pGWB17	Overexpression studies

Chapter 3. Characterization of MKP2 and DsPTP1 function

3.1 *In-silico* analysis of tissue-specific expression patterns of *MKP2* and *DsPTP1*

To gain insight into the potential functions of MKP2 and DsPTP1 in plant growth and development, publicly available gene expression patterns were obtained from the Arabidopsis eFP Browser 2.0. The *in-silico* tissue-specific expression patterns of *MKP2* and *DsPTP1* enabled the narrowing of the investigation to specific organs by focusing on investigating roles associated with the organs that show elevated or reduced expression levels for the two phosphatase genes.

The Arabidopsis eFP Browser 2.0, developed by Winter et al. (2007), is an online tool that allows for the visualization of DNA microarray data revealing the expression patterns of various genes in *Arabidopsis thaliana*. These expression patterns can be used to give insight into the functions of genes based on their specific expression patterns. Figure 2 shows a summary of the output obtained from the eFP Browser tool for *MKP2* and *DsPTP1*, respectively.

As shown in Figure 2 (and Supplementary Figure 1), both *MKP2* and *DsPTP1* were found to be expressed in similar plant organs and at similar stages of development, albeit at varying intensities. The collected data shows relative expression profiles among different tissues. *MKP2* is not strongly expressed in most tissues, and since all its relative tissue-specific expression levels are below two, they are not deemed significant. *MKP2* is however slightly more expressed in the carpel and the shoot apex compared to the median gene expression. In the flower, *MKP2* is slightly more expressed at developmental stage 9 (during flower development), at which point the expression gets progressively weaker throughout stages 10 and 11. At stage 12 of flower development, *MKP2* is not strongly expressed compared to the median gene expression. Additionally, it is moderately expressed throughout most stages of leaf development (from developmental stage 2 to the cauline leaf stage). *MKP2* is also represented in most stages of seed and cotyledon development (developmental stages 2 to 10). On the other hand, *DsPTP1* is most strongly expressed in the stamen of mature plants, as well as in mature pollen, with moderate expression in the shoot apex and seed stage. The intense relative expression level of *DsPTP1* in the mature pollen and stamen suggests that the potential developmental role of DsPTP1 could be occurring in these tissues and be related to reproductive function. *MKP2* did not display any notable expression in either the stamen or pollen of mature plants.

In a pattern similar to that of *MKP2*, *DsPTP1* is mostly represented in a few above-shoot tissues: the cauline, rosette, and senescent leaves, the cotyledon, and the flower, but not at significantly different levels. Both genes show overlapping expression patterns in developing organs (cotyledon, hypocotyl) and mature organs (internode and flower), but the relative gene expression levels in most organs (other than the stamen and mature pollen for *DsPTP1*) is close to one and therefore not deemed significant. According to the *in-silico* data, *DsPTP1* is expressed in flowers, and more specifically, the pollen and stamen, which might suggest potential roles of DsPTP1 in these reproductive tissues. Like *MKP2*, *DsPTP1* is expressed in most stages of seed and cotyledon development, but the expression levels are relatively close to the median gene expression. The expression of *DsPTP1* in the seed stages is consistent with previous reports of DsPT1 being involved in seed germination under osmotic stress (Liu et al., 2015).

It should be noted that *MKP2* and *DsPTP1* are differentially expressed in the pollen and stamen, which might suggest different roles, if any, of each phosphatase in these tissues. While *DsPTP1* is most strongly expressed in these tissues, *MKP2* is expressed at a relative level of less than one. Overall, *MKP2* is not intensely represented in any of the tissues; its relative expression levels remain close to one in the above-shoot tissues where it is the most expressed (flower, shoot apex, rosette and cauline leaves, carpel, and cotyledon). According to DNA microarray data, *DsPTP1* mRNA transcripts are more strongly expressed in the mature pollen and stamen compared to the median gene expression. It should also be noted that mRNA transcripts for regulatory genes could be expressed at lower levels due to tight regulation at the transcriptional level. Therefore, a lower level of transcript for a given gene does not necessarily indicate that the gene is unlikely to have a function in a given tissue.

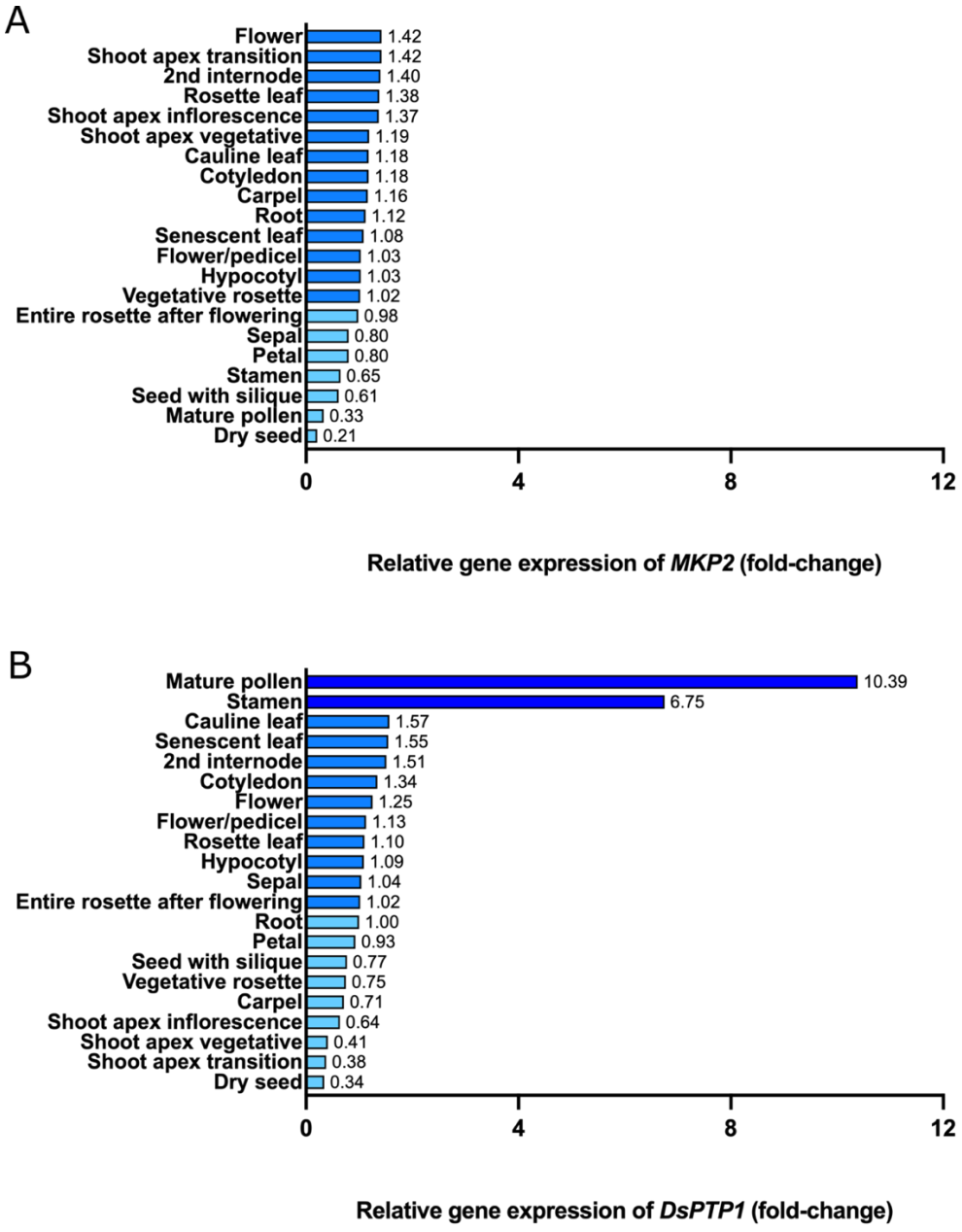


Figure 2. Relative tissue-specific gene expression of (A) *MKP2* and (B) *DsPTP1* in *Arabidopsis thaliana*. For each tissue, plotted relative expression is the ratio of gene expression level to the median gene expression level of the respective gene among all tissues. Cyan bars show a relative expression level lower than one, and dark blue bars show a relative expression level greater than two. Other bars show a relative expression level that is close to one. The data is based on the outputs obtained from the Arabidopsis eFP Browser 2.0 (Schmid et al., 2005; Winter et al., 2007).

3.2 Cellular protein localization of MKP2 and DsPTP1 at different stages of development

To gain further insight into the function of MKP2 and DsPTP1 in plant development, cellular protein localization studies were conducted. I used full-length MKP2-GFP and DsPTP1-GFP fusion constructs driven by the 35S constitutive promoter rather than the native promoters, as MKP2 and DsPTP1 levels under normal developmental conditions are very low. This information will be used gain functional insight into the two phosphatases.

To investigate the localization patterns of MKP2 and DsPTP1, independent Arabidopsis lines expressing each GFP-tagged phosphatase were generated. The constructs were designed so that the phosphatases would be fused with a GFP tag to allow for visualization of the localization patterns. Seeds were then sown, and the resulting seedlings were imaged by confocal microscopy at 3 days, 8 days, and 5 weeks post-germination. Col-0 and 35S::GFP seedlings under the control of the 35S promoter of the Cauliflower mosaic virus (CaMV), which is constitutively expressed in all cell types, were used as negative and positive controls, respectively.

At 3 and 8 days post-germination (dpg), the leaf epidermis, root elongation zone and root tip of seedlings of each line were imaged to obtain representative localization patterns for constitutively-expressed MKP2 and DsPTP1 proteins.

At 3 days post-germination, it is observed that MKP2 has a similar expression pattern to the positive control in the leaf epidermis and is effectively expressed in nearly every part of the tissue. Previous reports inferred that the cytoplasm and the plasma membrane are the cellular locations of the MKP2 protein product according to sequence similarity detection methods and a high throughput direct assay (nano-LC-MS/MS) (Marmagne et al., 2007). This is consistent with the biological component of MAPKs, which act in signal transduction, where they receive signals near the plasma membranes and elicit cellular responses by acting on transcription factors in the nucleus. MKPs negatively regulate MAPKs and are therefore co-localized with them. While the magnification used in these assays does not allow us to infer the subcellular localization of MKP2 and DsPTP1, the images show that MKP2 might be localized in the cytoplasm and plasma membranes of pavement cells and guard cells. This would however need to be further investigated by using higher magnifications that would allow for a better resolution. According to previous phylogenetic tree and sequence similarity detection methods, DsPTP1 is inferred to be expressed in the cytoplasm and the chloroplasts (Gaudet et al., 2011). However,

the micrographs show that 35S promoter-controlled DsPTP1 is not expressed in any of the observed tissues at 3 dpg and contrast with the GO annotations.

Overall, while characterizing the localization patterns of the MKP2-GFP and DsPTP1-GFP overexpression lines, I found that MKP2-GFP and DsPTP1-GFP primarily accumulated in specific tissues, unlike the *35S::GFP* line, where GFP fluorescence is detected in all cell and tissue types and developmental stages. This suggests that MKP2 and DsPTP1 may be degraded either post-transcriptionally or post-translationally in certain tissues during plant development.

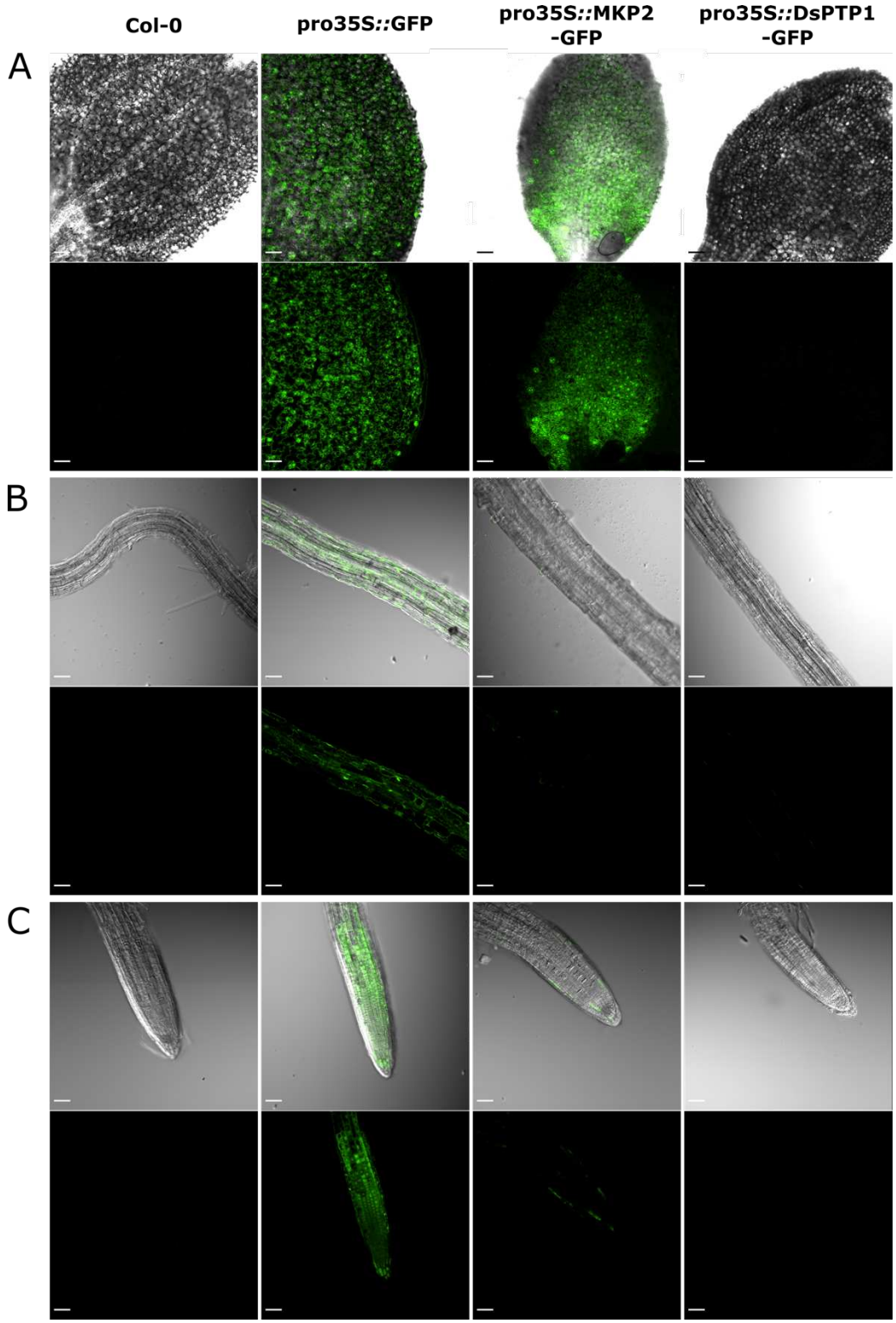


Figure 3. Cellular protein localization of GFP, MKP2-GFP, and DsPTP1-GFP proteins under the control of the 35S promoter in leaf epidermis and roots at 3 days post-germination. Confocal images of the GFP fluorescence of each protein in the (A) leaf epidermal tissues, (B) root elongation zone, and (C) root tip are shown. DIC (composite) and GFP channels were captured for each protein. Scale bars = 50 μm.

To further establish the expression pattern of constitutively-expressed MKP2 and DsPTP1 in relation to the developmental stage, the seedlings were imaged at 8 days post-germination (Figure 4).

At this stage, the *pro35S::GFP* plants exhibit GFP expression in nearly every part of the leaf epidermis, root elongation zone and root tip, including the cell membranes and nuclei. Since the GFP fusion constructs are controlled by the constitutive 35S promoter, the *pro35S::GFP* line is used as a positive control and a baseline for the amount of GFP signal that can be expected from the *pro35S::MKP2-GFP* and *pro35S::DsPTP1-GFP* lines.

The *pro35S::MKP2-GFP* line has a similar expression pattern as the *pro35S::GFP* line and shows a high-intensity signal in the leaf epidermis, root elongation zone, and root tip, which indicates MKP2 expression in these tissues. While conclusions cannot be drawn regarding endogenous subcellular localization due to the use of a constitutive promoter, the results suggest that MKP2 might be found in the plasma membranes, nucleus, and cytoplasm of cells of the leaf epidermis. It is, however, also found in the cells of the root elongation zone and root tip, which is not the case at 3 dpg. MKP2 is also expressed in the root elongation zone and root tip. Taken with the MKP2 expression patterns at 3 dpg, these results could indicate a stage-specific involvement of MKP2 in development at 8 dpg that might not present at the earlier stages of development, but this would need to be further investigated using native promoter-controlled transgenic lines.

This is contrasted with the expression pattern of constitutively-expressed DsPTP1, which is expressed in the root, but at a slightly lower intensity than MKP2. However, DsPTP1 is not expressed in the leaf epidermis at either stage of development.

While MKP2-GFP and DsPTP1-GFP are both expressed in the root elongation zone and near the root tip when driven by the 35S promoter, the fluorescence signals are noticeably weaker than for the *35S::GFP* line, which again suggests that MKP2 and DsPTP1 may be degraded post-transcriptionally or post-translationally in certain tissues. At the leaf epidermis, unlike DsPTP1, MKP2 is expressed at a similar intensity to *35S::GFP*, which could suggest that MKP2 does not get degraded post-transcriptionally or post-translationally at this developmental stage in this specific tissue.

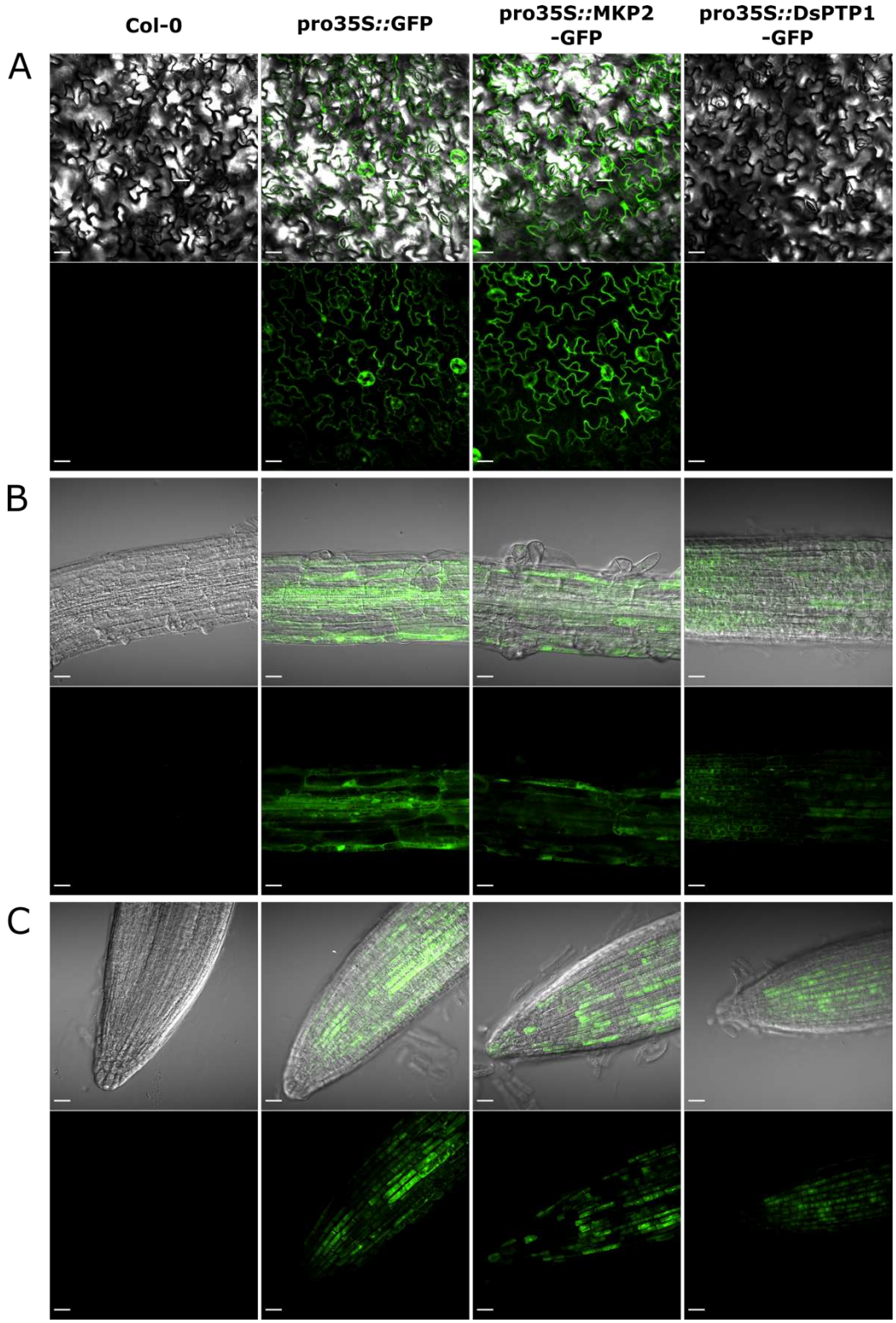


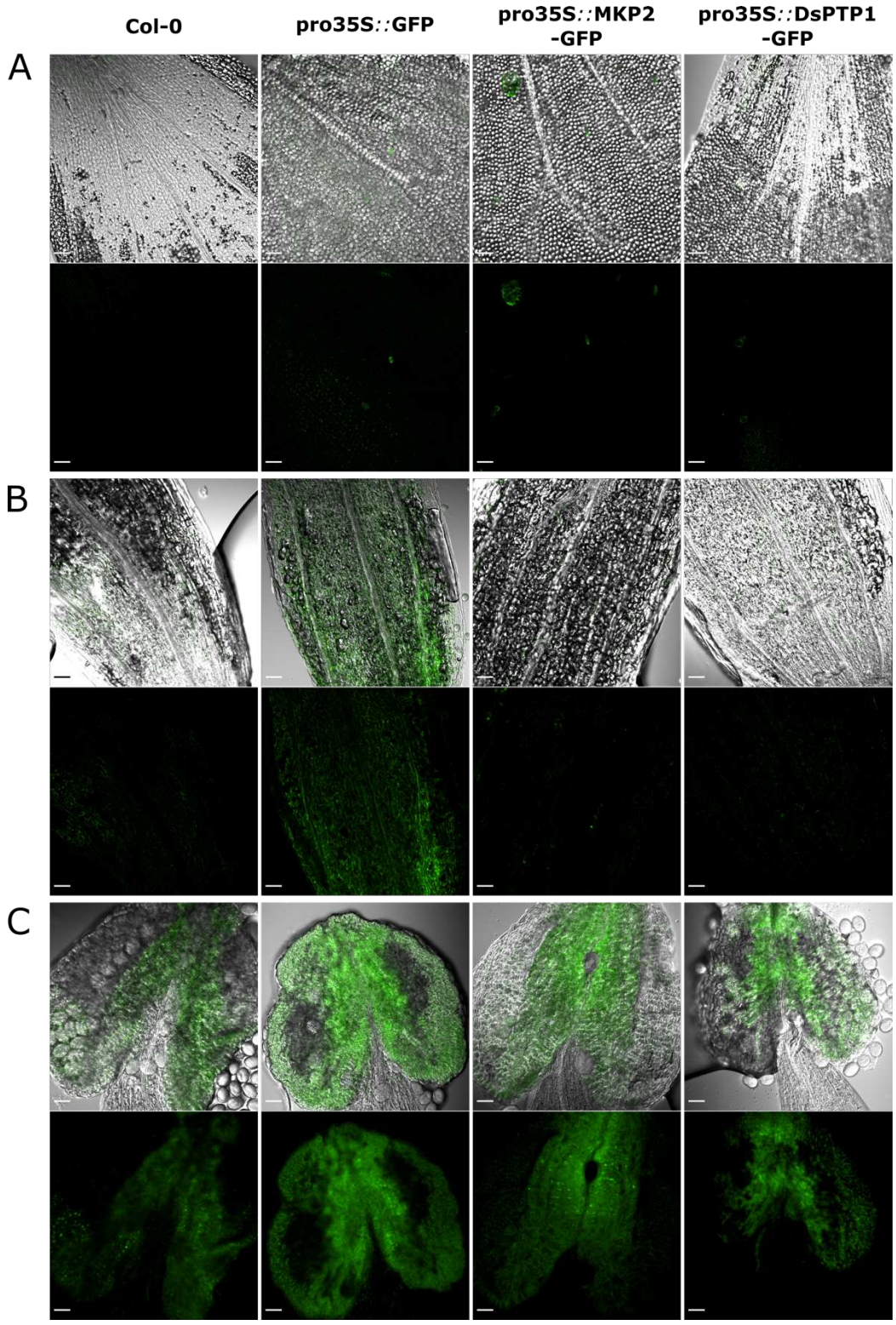
Figure 4. Cellular protein localization of GFP, MKP2-GFP, and DsPTP1-GFP proteins under the control of the 35S promoter in leaf epidermis and roots at 8 days post-germination. Confocal images of the GFP fluorescence of each protein in the (A) leaf epidermal tissues, (B) root elongation zone, and (C) root tip are shown. DIC (composite) and GFP channels were captured for each protein. Scale bars = 50 μm.

Seedlings were also transplanted and grown to near maturity (5 weeks) to observe the protein localization patterns in mature plant parts. At the mature stage, the reproductive tissues were prone to autofluorescence, which is indicated by a level of GFP signal in the wild-type control, which does not have any GFP fusion construct. The expression levels for the reproductive tissues are therefore compared with the basal signal level that is found in the control. At this later stage of development, MKP2 and DsPTP1 are generally either expressed at very low intensities comparable to the autofluorescence that is detected in the control samples, or not expressed. This can be seen in most of the reproductive organs that were observed (sepal, petal, filament, and carpel), which do not show any true expression of MKP2 and DsPTP1. The anther, however, exhibits expression levels that are slightly higher than the wild type, which could indicate weak expression at this stage of development. Overall, these results suggest that MKP2 and DsPTP1 are unlikely to be strongly expressed in the reproductive tissues. This is contrasted with the tissue-specific expression patterns at earlier stages of development, which suggest MKP2 expression in the observed vegetative tissues.

The data for transcript and protein localization patterns do not fully match, even when considering the use of the 35S promoter. Multiple factors such as post-transcriptional and translational regulation could explain this discrepancy. Post-transcriptional regulation mechanisms such as splicing can create different mRNA isoforms that have different localization patterns (Zhao et al., 2017). Translational and post-translational regulation mechanisms such as phosphorylation can affect the localization of proteins (Ramazi & Zahiri). It should also be noted that mRNAs are not necessarily all directly translated and are sometimes stored in cellular compartments until translation is triggered by specific signals. Another likely phenomenon that could explain the discrepancy is protein degradation, which could be due to regulatory mechanisms such as ubiquitination, or simply due to protein turnover caused by the proteins having shorter lifetimes in the cell (Li et al., 2017).

Taken together, the protein localization studies at 3 days, 8 days, and 5 weeks post-germination (Figures 3, 4 and 5) show that the MKP2 and DsPTP1 protein products could be localized in the vegetative tissues but are subject to post-transcriptional or post-translational degradation in certain tissues and at certain stages of development. The protein-level expression patterns suggest that MKP2 and DsPTP1 could play roles in vegetative development, but further studies

using native promoter-controlled transgenic lines should be conducted to add support to this possibility.



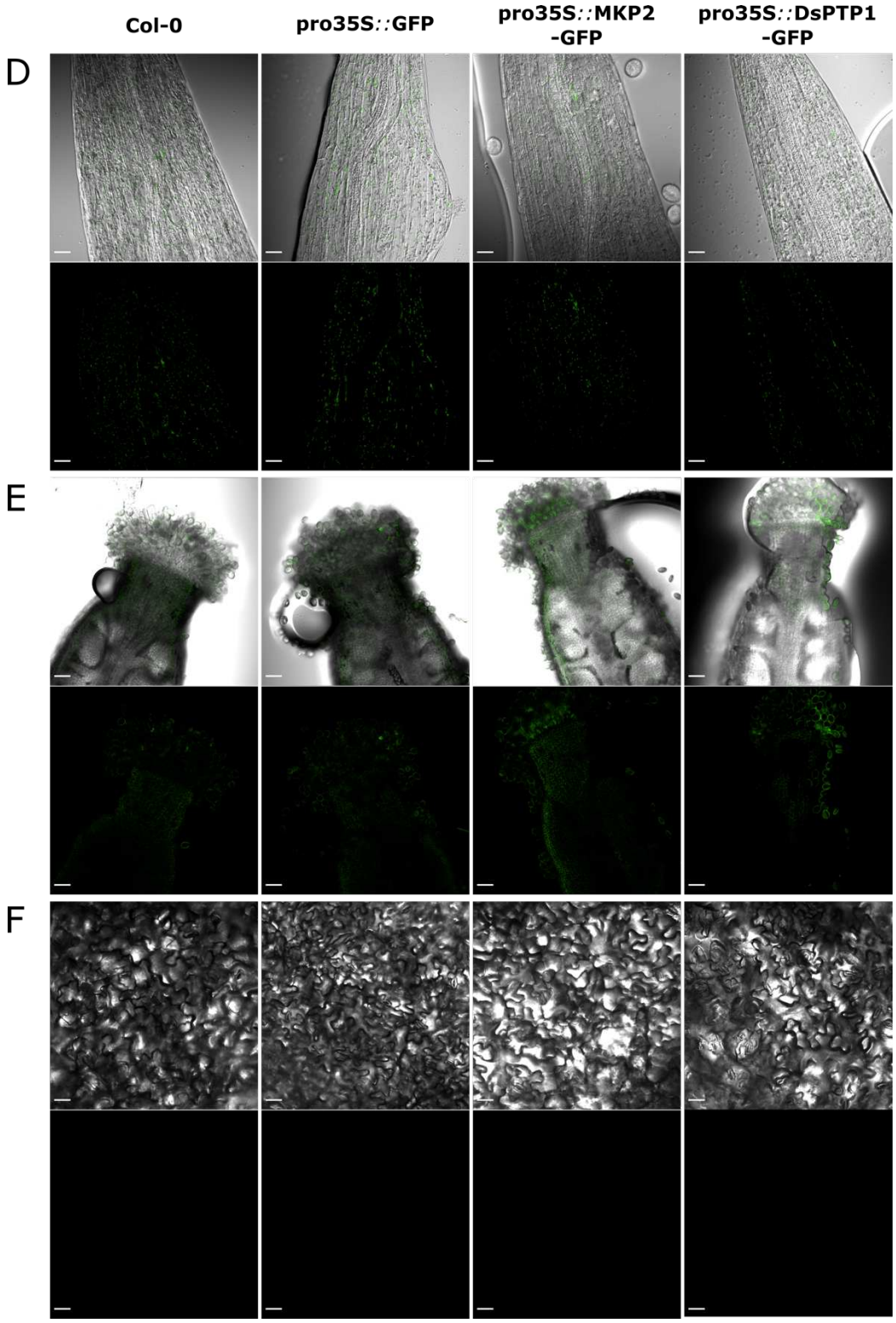


Figure 5. Cellular protein localization of 35S promoter-controlled GFP, MKP2-GFP, and DsPTP1-GFP proteins in reproductive organs and leaf epidermis at 5 weeks post-germination. Confocal images of GFP fluorescence of each protein in the (A) petal, (B) sepal, (C) anther, (D) filament, (E) carpel, and (F) mature leaf epidermis are shown. DIC (composite) and GFP channels were captured. Scale bars = 50 μm.

3.3 Silique seed segregation assay

Previously, 5-day-old *mkp2 dsptp1-1* seedlings were found to exhibit a drastic seedling-lethal albino phenotype that might be associated with defects in chloroplast development (Lee Lab, unpublished work). To further investigate the albinism that was initially found in *mkp2 dsptp1-1* plants at the seedling stage of development, and determine if it affects the seed developmental stage, a silique seed segregation assay was conducted.

Plants carrying the *mkp2-2 dsptp1-1/+* mutation were simultaneously grown with complementation lines (*proMKP2::MKP2* and *proDsPTP1::DsPTP1* in double mutant backgrounds) to near-maturity (6 weeks post-germination) to observe the seed phenotype in maturing siliques. The heterozygous double mutants (*mkp2-2 dsptp1-1/+*) were specifically used for their ability to reveal the genetic nature of the mutation via segregation that can be inferred from the resulting seed phenotypes (albinism or lack of albinism in a certain proportion of seeds). Native promoter-controlled complementation lines were also used to confirm that complementation also occurs at the seed stage of development in addition to the seedlings stage, which was previously observed in the laboratory.

Figure 6A-D shows the cross-section of the wild type, *mkp2-2 dsptp1-1/+*, and native promoter-controlled MKP2 and DsPTP1 complementation lines, respectively. Whereas the complementation lines exhibit silique and seed phenotypes identical to that of the wild type, where seeds all have a normal green appearance, *mkp2-2 dsptp1-1/+* siliques show albinism in the seeds at a ratio of 28.43% (Figure 6E, n=6). This segregation ratio is close to the Mendelian expectation of a 3:1 segregation ratio (non-albino to albino) for a monohybrid cross, which indicates that the white seeds are indeed *mkp2-2 dsptp1-1* seeds.

This result shows that the *mkp2-2 dsptp1-1* mutation might interfere with chloroplast development at the seed developmental stage but does not prevent proper embryo development since the seeds maintain the ability to germinate and grow to the early seedling stage, at which point the inability of the plant to efficiently perform photosynthesis causes seedling lethality.

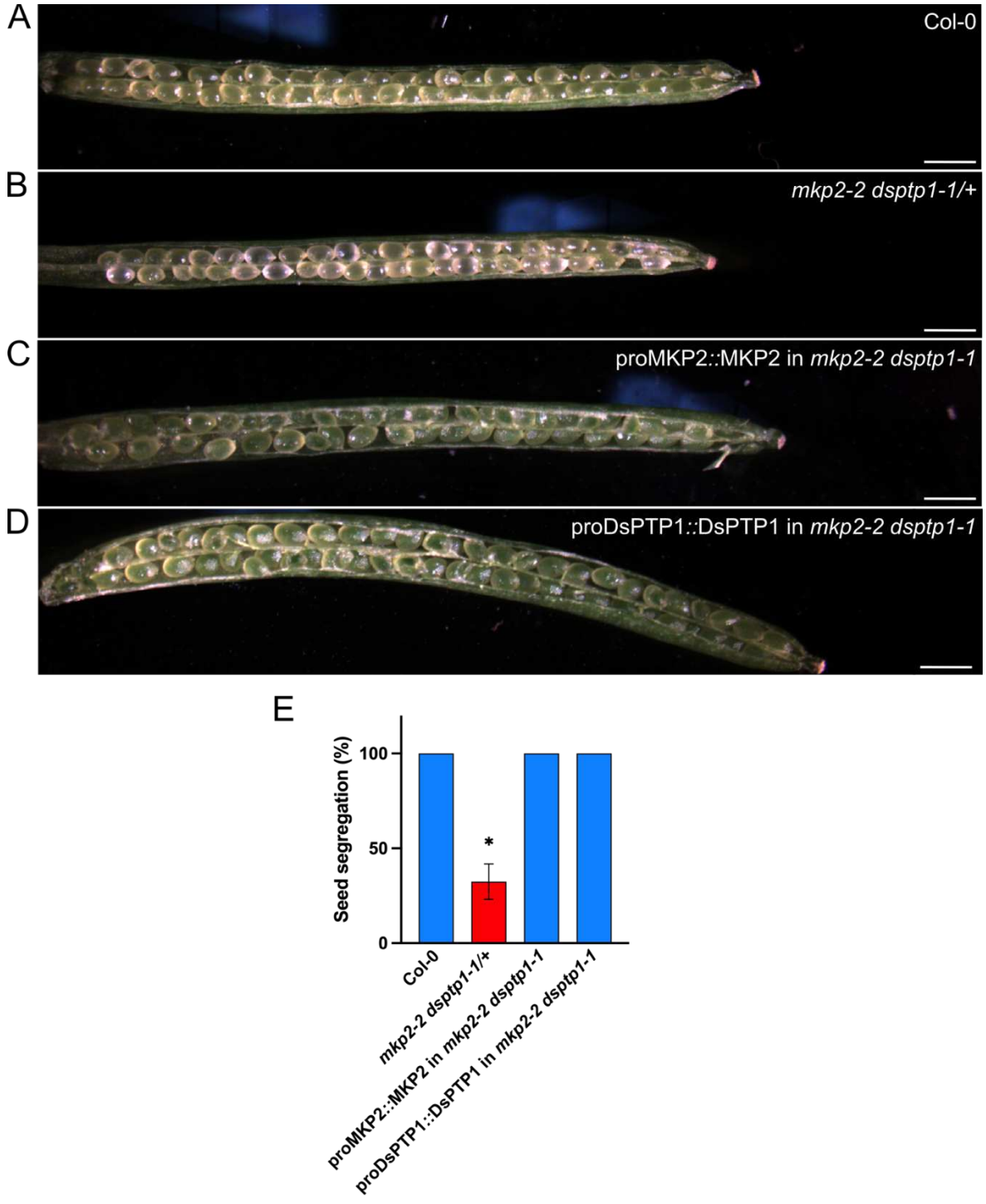


Figure 6. Seed silique segregation analysis in 6-week-old *Arabidopsis thaliana* plants. (A) Seeds in silique of Col-0 wild-type plant, (B) *mkp2-2 dsptp1-1/+* heterozygous plant, and (C) plants expressing a proMKP2*::*MKP2 or (D) proDsPTP1*::*DsPTP1 transgene in a double mutant background. The scale bars represent a length of one millimetre. (E) Segregation ratio of albinism in the siliques of each plant genotype (n=6). Scale bars each represent one millimetre.

3.4 Photosynthetic pigment quantification assay

Since albinism in plants is most often an indication of reduced photosynthetic pigment levels in normally green tissues, affecting proper chloroplast development, photosynthetic pigment quantification assays were conducted to investigate this possibility. Previous preliminary findings show that *mkp2-2 dsptp1-1* mutants exhibit a drastic albino phenotype that is not present in either single mutant (Lee Lab, unpublished work).

The photosynthetic pigment levels for chlorophyll *a*, chlorophyll *b*, and carotenoids were quantified. These are the primary photosynthetic pigments used to capture light for photosynthesis in Arabidopsis and most other plant species. The photosynthetic pigment levels were quantified in Col-0, *mkp2-2 dsptp1-1* and MKP2 and DsPTP1 overexpression lines controlled by the CaMV 35S promoter (three independent *pro35S::MKP2-GFP* and *pro35S::DsPTP1-4xMyc* lines, respectively) using the Lichtenthaler & Wellburn (1983) equations.

In this experiment design, the Col-0 wild type is used as a baseline for normal photosynthetic pigment levels found in wild-type Arabidopsis plants. Following one-way ANOVAs that detected a difference in means for each pigment group, Tukey's HSD tests were run within each group to determine the statistically significant samples. Loss-of-function *mkp2-2 dsptp1-1* plants, which exhibit an albino phenotype resulting from the double mutation for *MKP2* and *DsPTP1*, show severely reduced levels of the three pigments (Chl*a*, Chl*b,* and carotenoid) with a statistically significant difference. This result is also reflected in total chlorophyll levels (Chl*a* + Chl*b*).

Next, the photosynthetic pigment levels were assessed for MKP2 overexpression lines. Since the overexpression MKP2 lines are tagged with GFP, a relatively large protein that might skew the results, a GFP overexpression line also controlled by the CaMV 35S promoter (*pro35S::GFP*) was used as an additional control. To ensure the reproducibility of the results, three independent *pro35S::MKP2-GFP* lines were used.

As shown in Figure 7A, *pro35S::MKP2-GFP* lines do not show a significant increase in photosynthetic pigment levels in comparison to the wild type. While a slight increase can be seen for each of the lines and the *pro35S::GFP* control, the differences fall within the error range and are not deemed statistically significant.

Likewise, *pro35S::DsPTP1-4xMyc* lines do not show significant increases in photosynthetic pigment levels compared to the wild type (Figure 7B). Taken together, these results confirm that *mkp2-2 dsptp1-1* exhibits albinism due to a decrease in chlorophyll content that could stem from a defect in chlorophyll and chloroplast synthesis. Additionally, it can be posited that individually overexpressing MKP2 and DsPTP1 does not necessarily result in significant increases in chlorophyll and carotenoid pigment levels.

All in all, photosynthetic pigment levels are severely reduced in the *mkp2-2 dsptp1-1* mutant, but no obvious differences were found in the MKP2 and DsPTP1 overexpression lines.

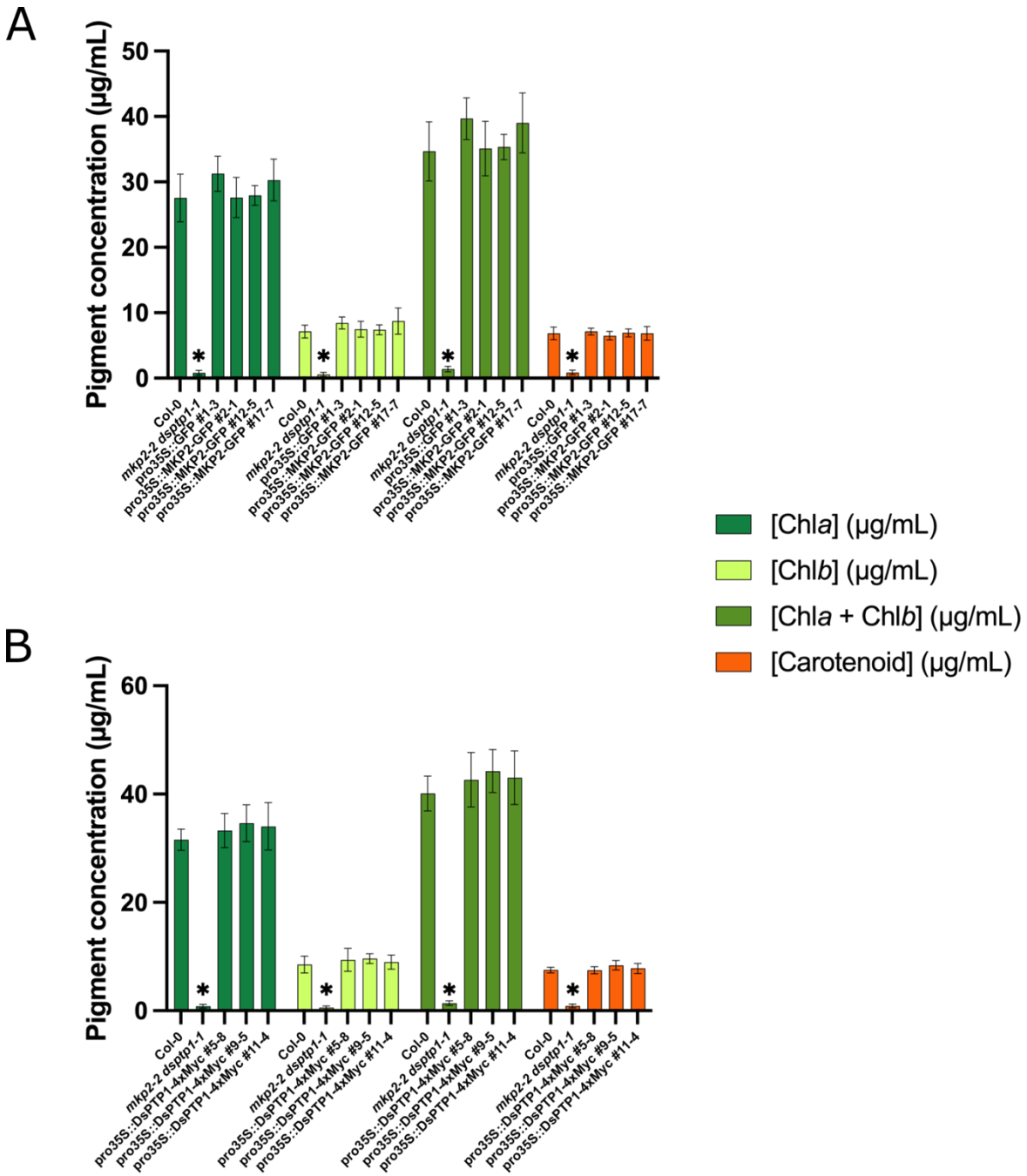


Figure 7. Photosynthetic pigment quantification assay. (A) Pigment concentrations were measured in Col-0, *mkp2-2 dsptp1-1*, *pro35S::GFP* and three independent transgenic MKP2 lines controlled by the CaMV 35S promoter. (B) Pigment concentrations were also independently measured in three independent *pro35S::DsPTP1* lines. Concentrations for chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid sampled from 5-day-old seedlings of each genotype were calculated using the Lichtenthaler & Wellburn (1983) equations, and the means are shown above. Following one-way ANOVAs, Tukey's HSD tests comparing the means of the pigment concentrations in the plant lines within each group ([Chl*a*], [Chl*b*], [Chl*a* + Chl*b*], and [carotenoid]) were conducted. Pigment concentrations associated with a p-value below the α level (0.05) are statistically significant and marked with an asterisk.

3.5 Primary root length assay in overexpression lines

The protein accumulation studies show the expression of MKP2 and DsPTP1 in roots (Figures 3 and 4), suggesting that there is a possibility that MKP2 and DsPTP1 function in root development at the earlier stages. To better assess the potential functions of MKP2 and DsPTP1 in vegetative development and to confirm the protein expression patterns in the roots that were observed at 3 and 8 days post-germination, root assays were performed in independent MKP2 and DsPTP1 overexpression lines at 10 dpg.

Seeds of each genotype were sown on ½ MS plates and grown vertically for 10 days after undergoing a cold/dark treatment for 3 days to ensure even growth of the seedlings.

Although MKP2 and DsPTP1 are both expressed in the root elongation zone and root tip at 8 dpg when controlled by the 35S promoter, the root length assays do not reveal any significant difference between the *pro35S::MKP2-GFP*, *pro35S::DsPTP1-GFP* lines and the wild-type seedlings (Figure 8A, B). While the root lengths of the overexpression lines are generally slightly larger than the wild type (except for *pro35S::MKP2-GFP* #5-6), the difference is not statistically significant according to a one-way ANOVA comparing the values for the overexpression lines to those of the control group (Col-0), as shown in Figure 8C. The statistical analyses that were performed rule out the possibility that an overexpression of MKP2 or DsPTP1 results in development-related phenotypes in the root.

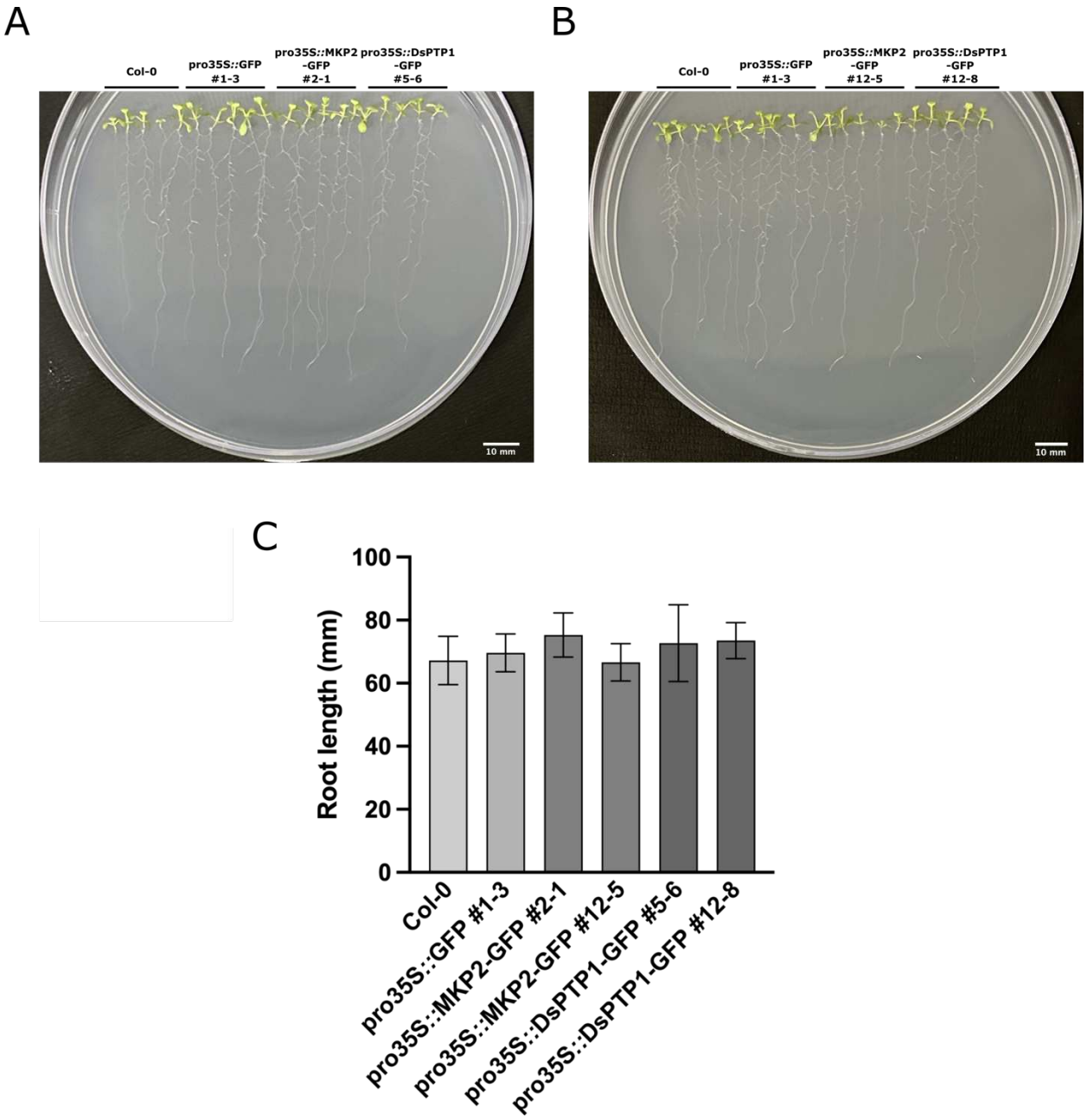


Figure 8. Primary root assay of overexpression lines (*pro35S::GFP*, *pro35S::MKP2-GFP*, *pro35S::DsPTP1-GFP*) at 10 days post-germination. (A, B) Shown are *pro35S::MKP2-GFP* and *pro35S::DsPTP1-GFP* seedlings from two independent lines on ½ MS plates after growing vertically. The seedlings were grown alongside Col-0 and *pro35S::GFP* controls. (C) The mean primary root lengths were computed and plotted on a bar graph. The standard deviation for each group is represented by error bars. Five seedlings of each genotype/plant line were grown per plate, and four plates were used in total (n=20).

3.6 Phenotypic assays of overexpression plants

To better assess the potential functions of MKP2 and DsPTP1 at the mature developmental stage, phenotypic assays were performed in different plant tissues of *MKP2* and *DsPTP1* overexpression lines. The plants were assayed at 8 days and 5 weeks post-germination, and the phenotypes were observed at the seedling and mature stages, and in the reproductive organs (inflorescences, flowers, and siliques).

Overexpression approaches are often used to add to loss-of-function approaches. Per the literature, single loss-of-function *dsptp1-1* and *mkp2-2* mutants both exhibit stress response-related phenotypes but were not previously found to exhibit any abnormal development-related phenotypes. Loss-of-function *dsptp1* mutants were previously shown to exhibit an increased germination ratio when subjected to osmotic stress (Liu et al., 2015), and *mkp2* mutants were shown to be hypersensitive to oxidative stress (Lee & Ellis, 2007).

As shown in Figure 9A, the seedlings exhibit a wild-type phenotype at 8 dpg under normal conditions. The mature plants also do not exhibit any abnormal phenotype and look like the Col-0 plants (Figure 9B). An overexpression of MKP2 and DsPTP1 does not seem to significantly affect the length of the shoots or other aspects of the mature plants, like silique length and number, which suggests that an overexpression of either phosphatase might not interfere with the function of MKP2 and DsPTP1 in plant development under the growth conditions of the assay. It should be noted that these lines are likely to show stress-related phenotypes under osmotic and oxidative stress as the *mkp2* and *dsptp1* mutants were previously shown to exhibit abnormal phenotypes under abiotic stress.

There are multiple factors that can explain the lack of abnormal phenotypes in the overexpression lines. One of the possibilities is that increasing the wild-type gene copies of either *MKP2* or *DsPTP1* does not lead to a gain-of-function phenotype as the gene phosphatases could still be able to properly carry out their functions at that specific level of overexpression.

In Figure 9C and D are shown the phenotypes of inflorescences, flowers, and siliques of mature Arabidopsis plants, including *MKP2* and *DsPTP1* overexpression lines. *MKP2* and *DsPTP1* overexpression lines are similar to the wild type and do not exhibit any abnormal phenotype in any of the observed tissues, which again suggests that the overexpression approach is not conclusive. The lack of abnormal phenotypes could be partially caused by the overexpression lines not necessarily having active versions of *MKP2* and *DsPTP1* despite their increased

expression. Phenotypic analysis using catalytically-active and catalytically-inactive versions of *MKP2* and *DsPTP1* at the same stages of development could be utilized to further explore the functions of MKP2 and DsPTP1 during plant development.

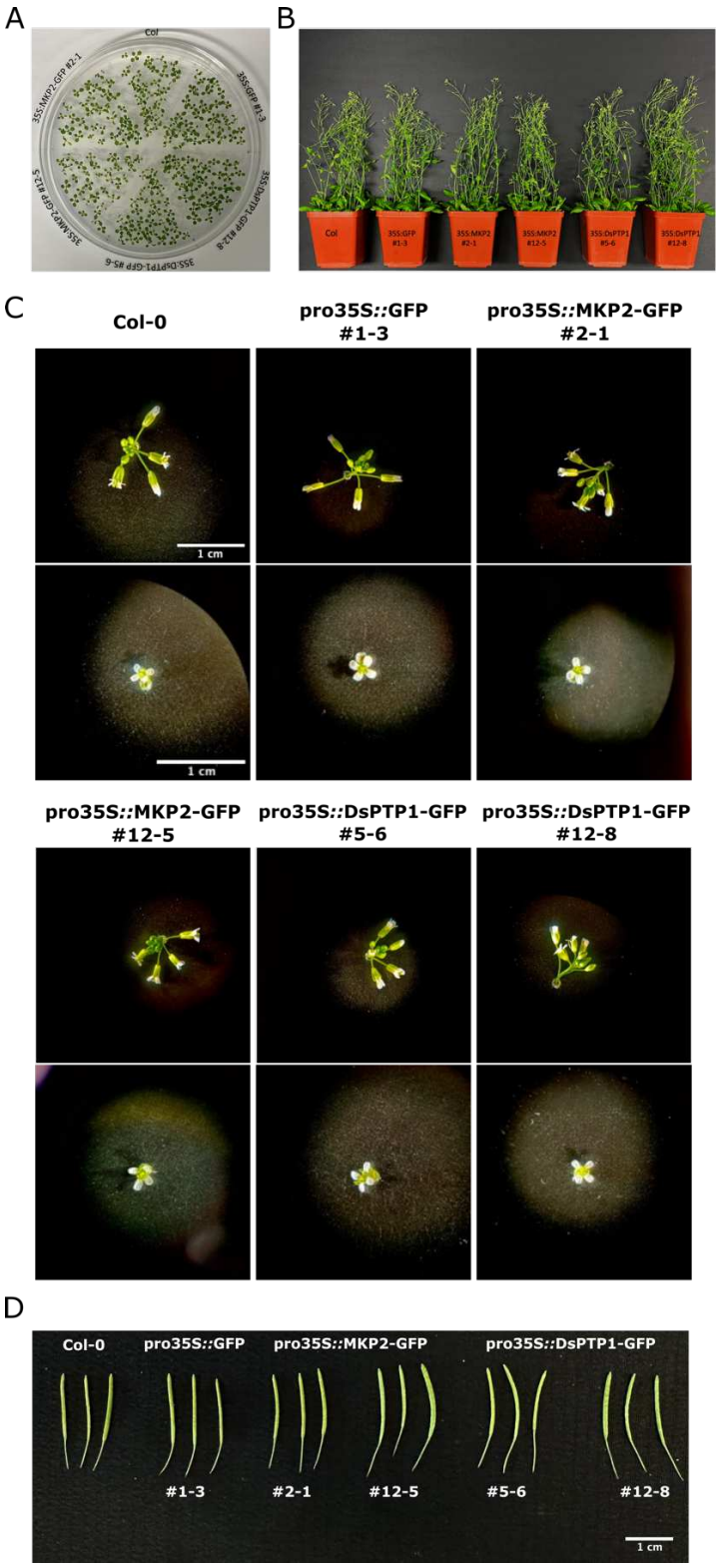


Figure 9. Phenotypic assays of overexpression lines (*pro35S::GFP*, *pro35S::MKP2-GFP*, *pro35S::DsPTP1-GFP*) at different stages of development. Shown are (A) the seedlings on ½ MS plate before transplanting, (B) plants at the mature developmental stage (5 weeks post-germination), (C) inflorescences and flowers, and (D) siliques.

Chapter 4. Protein interaction studies

4.1 Bimolecular fluorescence complementation assays in transient tobacco system

Following the characterization of MKP2 and DsPTP1 function, the next aim of the study is to identify MAPK interacting partners for MKP2 and DsPTP1. To investigate the potential physical protein–protein interactions between the protein products of the two phosphatases (MKP2 and DsPTP1) and the candidate MAPK substrates *in vivo*, bimolecular fluorescence complementation (BiFC) experiments were conducted.

Previous studies established that MKP2 physically interacts with MPK3 and MPK6 both *in vitro* and *in planta* (Lee & Ellis, 2007; Lumbreras et al., 2010). Phosphatase assays also show that MKP2 is capable of dephosphorylating phospho-MPK3 and phospho-MPK6, therefore acting as the enzyme in a phosphatase–kinase relationship (Lee & Ellis, 2007). The MAPKs (MPK3/4/6/8/15) tested through BiFC are those previously posited to be potential interacting partners for MKP2 and DsPTP1 through a literature search and previous preliminary experiments.

Gene constructs for MKP2 and DsPTP1 fused with the N-terminus (known as bait constructs) and constructs for the MAP kinases fused with the C-terminus (known as prey constructs) of split yellow fluorescent protein (YFP) were generated to test for physical interaction. The presence of interaction between two protein products can be established by observing whether fluorescence is restored, which can only happen when two protein interactors come physically together to reconstitute a full YFP molecule comprised of both N- and C-terminus halves, indicating the presence of physical interaction. YFP and DIC channels were each captured and overlayed to better assess the presence of fluorescent signals *in planta*.

Additionally, catalytically-inactive variants of the phosphatases ($MKP2^{C109S}$ and $DsPTP1^{C135S}$), which feature a modification in a conserved serine residue of the catalytic active site that was previously shown to disrupt chloroplast development, were fused with nYFP and cYFP (resulting in CIMKP2-nYFP and CIDsPTP1-nYFP) to test for interaction with the candidate MAPKs. The use of CIDsPTP1 and CIMKP2 constructs allows us to infer whether the phosphatase activities of MKP2 and DsPTP1, which are absent in CIMKP2 and CIDsPTP1, are required for physical interaction with target kinases *in vivo*. In this experiment, the empty cloning vectors fused with either the N- or C-termini (pBaTL-nYFP and pBaTL-cYFP, respectively) were used as the negative control, and they indeed do not give out any fluorescent

signal, which confirms the absence of interaction. The MKP2-nYFP–MPK6-cYFP combination, which has been previously established as a protein–protein interaction (Lumbreras et al., 2010), is used as the positive control.

MKP2-nYFP shows varying degrees of fluorescent signal when co-expressed with MPK4/6/8/15-cYFP, but not with MPK3-cYFP and DsPTP1-cYFP, which display no fluorescent signal (Figure 11A). While a strong fluorescent signal is observed when MKP2 is expressed with MPK6 (positive control), a somewhat weaker fluorescent signal is restored when it is expressed with MPK4. MPK8 and MPK15 also restore fluorescence when co-expressed with MKP2, but the signal is even weaker than what is observed in the MKP2-nYFP–MPK4-cYFP combination. Furthermore, while the restored fluorescence signal appears to be present in both plasma membranes and the nuclei of cells for the MKP2-nYFP–MPK4-cYFP and MKP2-nYFP–MPK6-cYFP combinations, a fluorescent signal is exclusively present in plasma membranes for the MKP2-nYFP – MPK8-cYFP and MKP2-nYFP – MPK15-cYFP combinations. This observation could give us insight into the localization of the protein products of MKP2 and the kinases themselves, but this would need to be further investigated. Taken together, these results allow us to posit that MKP2 interacts with MPK4, MPK6, MPK8, and MPK15 but at varying levels of strength. The physical interaction is strongest with MPK6, followed by MPK4 to a lesser extent, and weakest with MPK8 and MPK15. The lack of interaction of MKP2 with MPK3 is not consistent with a previous report showing strong interaction when co-expressed (Lumbreras et al., 2010). Since the previous study utilized different split-GFP vectors, the use of different vectors combined with the influence of the orientation of fusion tags (whether they are attached to the N- or C- terminus) can impact protein folding and, consequently, the resulting interactions, and might explain the discrepancies.

CIMKP2-nYFP shows identical results to that of MKP2-nYFP (Figure 11B); it interacts most strongly with MPK6-cYFP and MPK4-cYFP and gives out a relatively lower fluorescence signal strength when expressed with MPK8-cYFP and MPK15-cYFP. This result suggests that a functional catalytic domain is not required for normal physical interaction of the MKP2 protein product with potential kinase substrates, as the catalytically-inactive variant of the protein shows the same interaction patterns and affinities as its fully functional counterpart. According to BiFC, MKP2 and its catalytically-inactive variant do not physically interact with MPK3 and do not physically interact with DsPTP1.

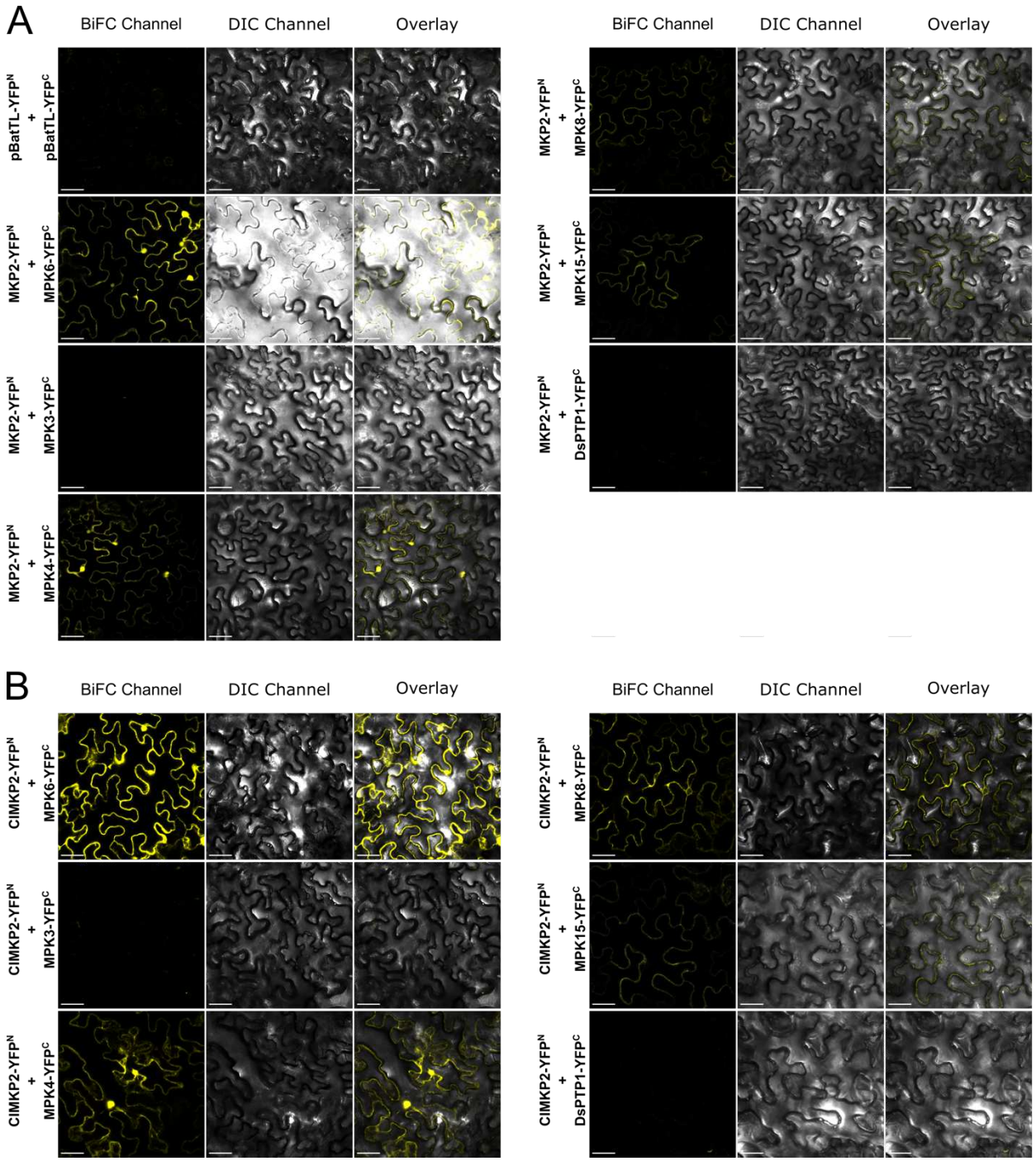


Figure 10. Bimolecular fluorescence complementation (BiFC) analysis to test for physical interaction between MKP2/CIMKP2 and candidate kinases. The YFP, DIC, and compound channels are shown. (A) BiFC testing for interactions between the MKP2-nYFP bait construct and prey constructs for candidate kinase substrates, and (B) CIMKP2-nYFP bait construct and kinase constructs. Interaction with DsPTP1-cYFP is also tested for MKP2-nYFP and CIMKP2-nYFP. The PBaTL-nYFP + pBaTL-cYFP combination is used as a negative control, and the MKP2-nYFP + MPK6-cYFP combination is used as a positive control. Scale bars = 50 μm.

DsPTP1, which was also tested for physical interaction with the candidate MAPKs, currently has no known MAPK interactor but is known to dephosphorylate and inactivate MPK4 in *in-vitro* phosphatase assays, which reflects an enzyme–substrate relationship (Gupta et al., 1998). However, the DsPTP1–MPK4 substrate relationship is not associated with any of the biological functions of DsPTP1 and the nature of the relationship has not been further described.

As shown in Figure 12A, DsPTP1 shows significantly different results when compared with MKP2. While strong to low levels of interaction for MKP2-nYFP were confirmed with MPK4/6/8/15-cYPF, only two of the DsPTP1-nYFP combinations exhibit any fluorescent signal. DsPTP1-nYFP gives out a weak fluorescent signal when co-expressed with MPK4-cYFP and MPK8-nYFP. The signal is significantly weaker than what is observed for any of the positive MKP2 – kinase interactions. The presence of physical interaction with MPK4, albeit weak, is consistent with a previous report of DsPTP1 and MPK4 having an enzyme–substrate relationship (Gupta et al., 1998), where DsPTP1 would come in close proximity to MPK4 in order to dephosphorylate and inactivate it. However, there is currently no published evidence of physical interaction between DsPTP1 and MPK4. DsPTP1-nYFP also exhibits some signal restoration when expressed with MPK6. This suggests that DsPTP1 could potentially interact with MPK6, which could partially explain its redundant function shared with MKP2, which also interacts with MPK6 in the present BiFC assays.

In a similar manner to that of DsPTP1-nYFP, CIDsPTP1-nYFP, the catalytically-inactive variant of the phosphatase, exhibits weak fluorescence when co-expressed with MPK4, MPK6 and MPK8 (Figure 12B). The disruption of the catalytic domain in CIDsPTP1 (and CIMKP2) does not affect the signal patterns and suggests that the phosphatase activity of DsPTP1 (and MKP2) is not required for the phosphatases to establish physical interaction with interacting partners.

Taken together, these results suggest that DsPTP1 and CIDsPTP1 do not physically interact with MPK3 but show a weak level of physical interaction with MPK4 and MPK8. The signal that is observed for the DsPTP1/CIDsPTP1 – MPK4 combinations is consistent with a previously published phosphatase assay but would need to be further investigated. MPK6 and MPK8 are not currently known to physically interact with DsPTP1 and are not known to be substrates of DsPTP1.

As shown in Figure 11, the MKP2-nYFP + DsPTP1-cYFP and CIMKP2-nYFP + DsPTP1-cYFP combinations display a lack of interaction. This suggests that DsPTP1 also does not physically

interact with MKP2, as shown by the lack of fluorescent signal when co-expressed, which rules out the possibility of MKP2 and DsPTP1 being interacting partners and is consistent with the initial hypothesis that MKP2 and DsPTP1 might function redundantly.

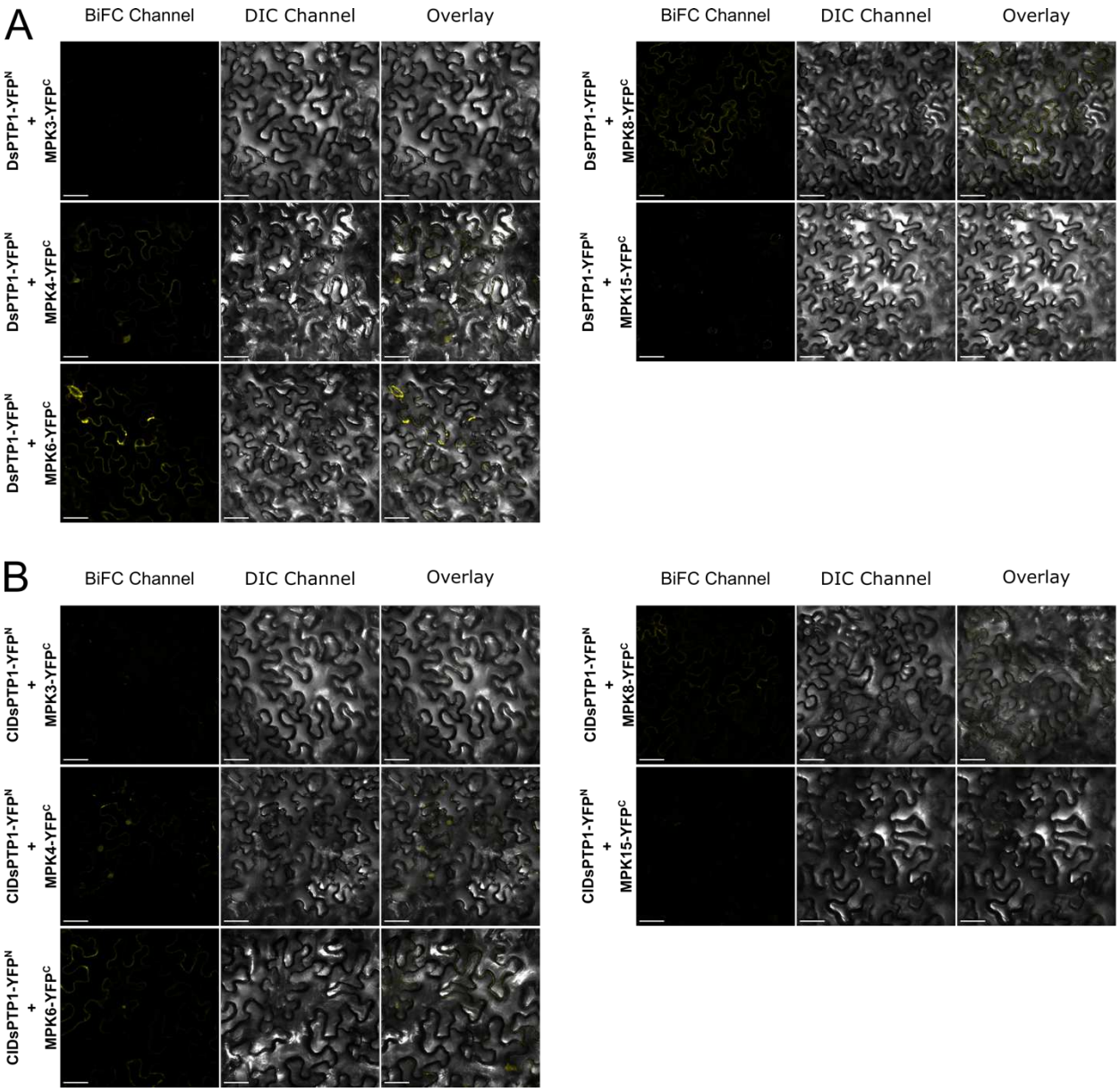


Figure 11. BiFC analysis to test for physical interaction between DsPTP1/ClDsPTP1 and candidate kinases. The YFP, DIC, and compound channels are shown. (A) BiFC testing for interactions between the DsPTP1-nYFP bait construct and kinase constructs for candidate kinase substrates, and (B) ClDsPTP1-nYFP bait construct and kinase prey constructs. The PBaTL-nYFP + pBaTL-cYFP and MKP2-nYFP + MPK6-cYFP combinations shown in Figure 11 were used as the negative and positive controls, respectively. Scale bars = 50 μm.

4.2 Yeast two-hybrid screens

To further investigate potential MKP-MAPK interactions, a set of Y2H experiments was designed. Plasmids for MKP2 and DsPTP1 and their catalytically-inactive variants (CIMKP2 and CIDsPTP1, respectively) fused with the DNA binding domain (BD) of the GAL4 transcription factor were generated. Likewise, constructs for the candidate kinases (MPK3/4/6/8/15) fused with the GAL4 activation domain (AD) were generated and co-transformed into AH109 strain yeast cells with their respective bait construct to test for physical interaction. The yeast two-hybrid system only allows for transcription of the upstream reporter gene (*HIS3*) if the transcription is activated by the reconstitution of the complete GAL4 transcription factor (including the BD and AD domains), which can only happen if physical interaction occurs between the prey and bait constructs. Selective minimal medium plates lacking histidine are used to determine the presence or absence of interaction. Yeast cells that carry a functional transcription factor through the interaction of bait and prey proteins can normally synthesize histidine and grow on minimal media. On the other hand, cells that do not exhibit physical interaction between their transformants are auxotrophic for histidine and do not grow on media lacking the nutrient. 3-Amino-1,2,4-triazole (3-AT) is a competitive inhibitor of the HIS3 reporter gene product and can be used to test for the auto-activation of bait constructs.

Because MKP2 showed auto-activation in the Y2H screens, minimal yeast plates with added 3-AT were used. Using the lowest 3-AT concentration where auto-activation does not occur allows for the elimination of auto-activation from the experiment design while only capturing true interactions that reconstitute the full transcription factor.

As shown in Figure 13A, MKP2 does not show any true interaction in the Y2H screens. While transformed yeast cells grow on selective SD/-Leu/-Trp/-His medium, they do not grow on relatively low 3-AT concentration plates (1 mM or 2.5 mM 3-AT), which yeast cells transformed with true interacting partners should relatively easily withstand, which confirms that the false positives seen on the SD/-Leu/-Trp/-His plates are likely due to the reporter gene being activated by auto-activators (Dreze et al., 2010). Results for MKP2-transformed yeast cells grown on SD/-Leu/-Trp/-His are therefore not accurate unless combined with the results for the same cells grown on 3-AT plates, which account for auto-activation. According to these Y2H screens, MKP2 does not interact with any of the kinases in the yeast system.

Next, the DsPTP1 – kinase combinations were tested and found to show similar results (Figure 13B) but with the absence of auto-activation. While the transformation control (grown on SD/-Leu/-Trp medium) exhibits cell growth, indicating properly transformed yeast cells, the selective plate does not allow for the growth of yeast cells transformed with any of the DsPTP1 – kinase combinations. This contrasts with a previous report of MPK4 serving as a target substrate for DsPTP1 (Gupta et al., 1998), and the BiFC data, which shows a low-intensity signal for DsPTP1 when co-expressed with MPK4, MPK6 and MPK8. It should be noted that Y2H is an *in-vitro* approach and does not test for physical interactions under native biological conditions.

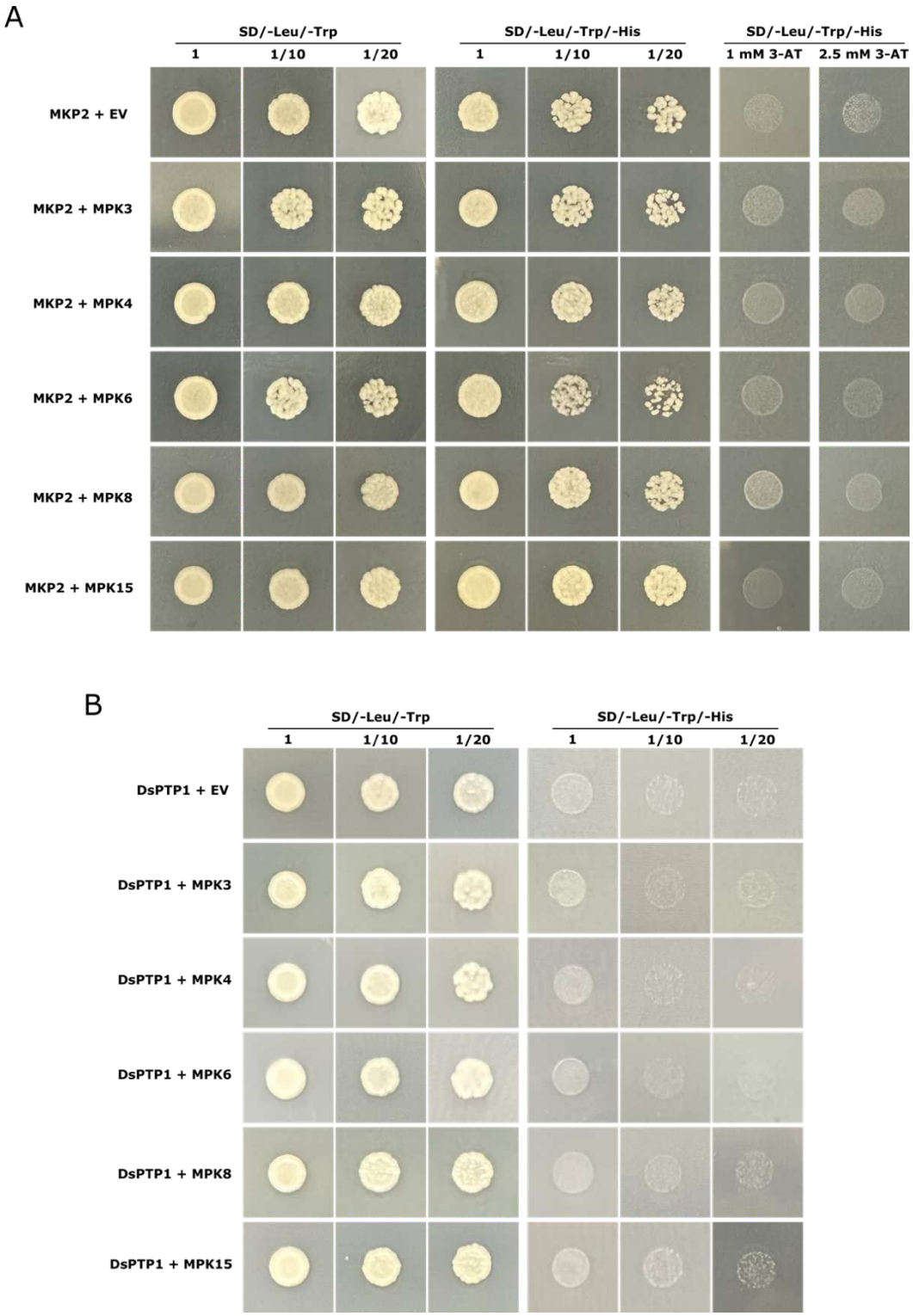


Figure 12. Y2H screening. (A) MKP2 and (B) DsPTP1 interactions with candidate MAPK prey constructs were tested. Shown are the transformed yeast colonies grown on growth (SD/-Leu/-Trp) and selective (SD/-Leu/-Trp/-His) media. Since MKP2 combinations resulted in auto-activation of the reporter gene, 1 mM and 2.5 mM 3-AT plates were used to validate the interactions. Plated colonies are indicated by their dilution factor for the SD/-Leu/-Trp and SD/-Leu/-Trp/-His plates.

Chapter 5. Discussion

5.1 MKP2 and DsPTP1 redundantly control chloroplast development.

Previous unpublished data from our research group suggest that MKP2 and DsPTP1 might work in a functionally redundant manner in aspects of plant development in Arabidopsis. It was found that the double loss of function of the two phosphatases resulted in a seedling-lethal albino phenotype that was a result of lower chloroplast and chlorophyll production (Lee Lab, unpublished work). This is also indicated by the photosynthetic pigment quantification assays, which show severely reduced pigment levels in *mkp2-2 dsptp1-1* double mutant seedlings and are consistent with previous findings (Figure 7).

To further investigate and characterize the functions of MKP2 and DsPTP1, protein localization and phenotypic assays (in siliques, reproductive organs, and roots) were performed. As shown in Figures 3 and 4, the early stages of development (from seed to seedling) are characterized by an albino phenotype resulting from severely reduced chlorophyll levels that indicate a possible defect in chloroplast biogenesis. In mutant seeds and seedlings, the albino phenotype is present at the expected Mendelian phenotypic ratio of 3:1 (non-albino to albino) for a monohybrid cross, which indicates that the albino phenotype is indeed a result of the disruption of the *MKP2* and *DsPTP1* genes (Figure 6E). While double mutant plant lines are still able to undergo seed development and germinate, they will be limited by seedling lethality, which occurs shortly after 5 days post-germination.

Moreover, protein localization studies (Figures 3–5) show that the translational fusion of *pro35S::MKP2* and *pro35S::DsPTP1* are generally not expressed in the same tissues, with the exception of the roots. While MKP2 is found in most tissues in the seedling, DsPTP1 is generally only found in the roots. The lack of spatial overlap in their expression indicates that MKP2 and DsPTP1 are unlikely to interact physically, and this is also corroborated by physical interaction studies, although differential protein degradation or technical issues with the *pro35S::DsPTP1* construct might be affecting the results. As shown in Figures 10 and 11, no fluorescent signal was detected for the MKP2-nYFP + DsPTP1-cYFP combinations in BiFC assays, which further validates the expression patterns seen in the protein localization studies and supports the possibility that MKP2 and DsPTP1 do not interact physically.

While the magnification used in the protein localization studies does not allow for concluding at the subcellular level, the transcriptional fusions of *pro35S::MKP2* and *pro35S::DsPTP1* seem to

be expressed in the nucleus and plasma membrane (Figures 3–5), which is expected for phosphatases as they mostly function in regulating MAPKs in the periphery of the cell and allowing their substrates to elicit cell responses through transcription factors in the nucleus by fine-tuning dephosphorylation and deactivation of their target substrates. These results would need to be further investigated using phosphatase constructs controlled by their respective native promoters.

A potential redundant function of MKP2 and DsPTP1 in plant development at the early stages (Lee Lab, unpublished work; Figure 6) might suggest that MKP2 and DsPTP1 could function in the same pathway and have similar target substrates. While MKP2 and DsPTP1 might be involved in the novel pathway along with some of the candidate MAPKs used in this study, they could also be partially part of a different pathway. Additionally, MKP2 targets MPK3 and MPK6 as interacting partners but might also target MPK8 and MPK15 for interaction and dephosphorylation, as evidenced by positive BiFC interactions (Figure 10). Through BiFC, DsPTP1 was found to interact with MPK4, MPK6 and MPK8. Phosphatase assays can be performed to further explore this possibility and identify substrates of each MKP.

Furthermore, it can be posited that the involvement of MKP2 in this pathway might at least partially involve the ABI4 transcription factor, which was previously shown to be positively regulated by MPK3 and MPK6 as part of its role in the transcription of *LHCB* genes, which are involved in multiple aspects of chlorophyll and photosynthetic function (Guo et al., 2016).

5.2 MKP2 and DsPTP1 might have roles in plant growth and development.

It was previously found that MKP2 and DsPTP1 work together to allow for proper development at the seedling stage of development (Lee Lab, unpublished work). The results generated in this study suggest the redundancy of MKP2 and DsPTP1 in controlling aspects of chloroplast biogenesis but also show that they might be involved in other aspects of plant growth and development, although this second possibility would need to be further investigated.

As shown in Figure 9, overexpression studies in MKP2 and DsPTP1 transgenic plants reveal no abnormal phenotype in any of the studied vegetative and reproductive tissues despite MKP2 being expressed in most tissues at the earlier stages of development (Figures 3 and 4). While this result might suggest that MKP2 and DsPTP1 are only involved in the early stages of plant development, it does not rule out the possibility that MKP2 and DsPTP1 might also be involved

in the later stages of plant development. As previously mentioned, phenotypic analysis using catalytically-active versions of *MKP2* and *DsPTP1* would be informative and allow us to further explore the functions of MKP2 and DsPTP1 during plant development.

Since *pro35S::MKP2* and *pro35S::DsPTP1* are both expressed in roots at 8 dpg according to protein localization studies (Figure 4), these tissues were further examined at the same stage of development. As shown in Figure 8, MKP2 and DsPTP1 overexpression does not result in any abnormal primary root phenotypes. However, this result only reflects the phenotypes associated with MKP2 and DsPTP1 overexpression in roots under experimental conditions and not necessarily under natural conditions, where overexpression of phosphatases could result in abnormal root phenotypes.

Taken with previous preliminary evidence, the phenotypic assays are not conclusive regarding the involvement of MKP2 and DsPTP1 in the later stages of development. The seed development assay taken with previous preliminary data of the double mutant phenotype in seedlings, however, indicates that MKP2 and DsPTP1 are likely involved in the earlier stages of development (seed and seedling stage), where their disruption results in a chloroplast-related seedling-lethal phenotype that first appears at the seed stage (Figure 6).

5.3 MKP2 and DsPTP1 physically interact with specific MAPKs.

Previous research in the field has established MAPK interactors for both MKP2 and DsPTP1. MKP2 was previously reported to dephosphorylate and physically interact with MPK3 and MPK6 as part of its role in regulating the oxidative stress response and immunity in response to pathogens (Lee & Ellis, 2007; Lumbreras et al., 2010). Although DsPTP1 has been found to have the ability to dephosphorylate and inactivate MPK4 (Gupta et al., 1998), this possible enzyme–substrate relationship is not linked to either of the two known functions of DsPTP1 (regulation of seed germination and ABA accumulation under osmotic stress) and has not been replicated under native biological conditions.

To further investigate the nature of their potential interactions with MAPKs, MKP2, and DsPTP1 were tested for their ability to physically bind to candidate MAPKs (MPK3/4/6/8/15) selected based on evidence found in the literature and through preliminary assays. BiFC and Y2H, the two physical interaction approaches that were utilized, partially reveal interactions for both MKP2 and DsPTP1. In accordance with previous research, MKP2 is found to physically interact

with MPK6, but also MPK4, MPK8, and MPK15, which have not previously been reported to interact with MKP2. It should also be noted that no interaction of MKP2 with MPK3 was detected in the BiFC experiment, which is not consistent with previous *in-vivo* studies that establish MPK3 as a main interactor of MKP2, along with MPK6 (Lee & Ellis, 2007). Although DsPTP1 is found to give out a fluorescent signal when co-expressed with MPK4, MPK6 and MPK8, the YFP signals are of varying intensities and are generally lower than those found for MKP2 interactions. While DsPTP1 might be involved in regulating some of the same MAPKs as MKP2 (MPK4/6/8 but not MPK15), the strengths of the interaction are generally lower for DsPTP1.

On the other hand, Y2H experiments contrast with this finding and do not show any DsPTP1 interaction with any of the tested MAPKs, as it shows no yeast growth on selective media when combined with any of the candidate MAPKs, despite a previous report of a possible enzyme–substrate relationship with MPK4. It should however be noted that Y2H and BiFC are approaches done in different biological systems (yeast cells and tobacco leaf tissue). The difference in cellular context between BiFC, which tests for interactions *in planta* in a system that is similar to the native environment of Arabidopsis proteins, and Y2H, which represents a foreign *in vivo* environment, explains the contrasting results produced through each method. Additionally, Y2H screens are often used to validate and add support to other interaction studies but are not commonly used on their own to confirm the possibility of physical interaction. MKP2 does not show any interaction in the Y2H screens but is shown to interact with MPK4, MPK6, MPK8 and MPK15 in BiFC assays, which suggests *in-planta* interaction under biological conditions. To confirm the results obtained *in planta* and address the contrasting nature of the Y2H data, *in-vitro* pull-down assays using recombinant proteins could be conducted to further test for physical interaction in an environment that lacks the foreign cellular context of Y2H.

Although MKP2 and DsPTP1 might have some degree of overlap in their MAPK interactors (namely MPK4, MPK6 and MPK8, even though the YFP signals are comparatively weaker in DsPTP1 combinations), the possibility that they might at least partially function in independent pathways cannot be ruled out.

Furthermore, the possible involvement of the phosphatase activities of MKP2 and DsPTP1 in their role in plant growth and development was also investigated throughout the study. It is known that the phosphatase activities of MKP2 and DsPTP1 are each required for their proper

function; as our research group previously found, plants expressing catalytically-inactive variants of MKP2 and DsPTP1 exhibit phenotypes identical to that of the *mkp2-2 dsptp1-1* seedlings, indicating that non-functional state of the catalytic domain in these constructs indeed disrupts the pathway (Lee Lab, unpublished work), in turn affecting chloroplast development and causing seedling lethality. While the proper function of the phosphatases is affected by the disruption of their catalytic domains via the modification of a conserved serine residue in the active site, BiFC reveals that the catalytic domain of either of the phosphatases is not involved in their ability to bind to their MAPK interactor(s), as MKP–MAPK combinations using catalytically-inactive variants of the phosphatases show the same interactions and interactions strengths as their wild-type counterparts (Figures 10B and 11B). This suggests that catalytically-inactive variants of MKP2 and DsPTP1 maintain the ability to bind their MAPK substrates but are unable to carry out their function by dephosphorylating their targets, owing to the disruption of the catalytic active site. Thus, the phosphatase activity is likely not required for the physical interaction of either phosphatase with its target substrate(s).

References

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F. M., & Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature*, *415*(6875), 977–983. https://doi.org/10.1038/415977a

Bartels, S., Anderson, J. C., González Besteiro, M. A., Carreri, A., Hirt, H., Buchala, A., Métraux, J.-P., Peck, S. C., & Ulm, R. (2009). MAP KINASE PHOSPHATASE1 and PROTEIN TYROSINE PHOSPHATASE1 Are Repressors of Salicylic Acid Synthesis and SNC1-Mediated Responses in *Arabidopsis*. *The Plant Cell*, *21*(9), 2884–2897. https://doi.org/10.1105/tpc.109.067678

Bazin, J., Mariappan, K., Jiang, Y., Blein, T., Voelz, R., Crespi, M., & Hirt, H. (2020). Role of MPK4 in pathogen-associated molecular pattern-triggered alternative splicing in Arabidopsis. *PLOS Pathogens*, *16*(4), e1008401. https://doi.org/10.1371/journal.ppat.1008401

Beckers, G. J. M., Jaskiewicz, M., Liu, Y., Underwood, W. R., He, S. Y., Zhang, S., & Conrath, U. (2009). Mitogen-Activated Protein Kinases 3 and 6 Are Required for Full Priming of Stress Responses in *Arabidopsis thaliana*. *The Plant Cell*, *21*(3), 944–953. https://doi.org/10.1105/tpc.108.062158

Bergmann, D. C., Lukowitz, W., & Somerville, C. R. (2004). Stomatal Development and Pattern Controlled by a MAPKK Kinase. *Science*, *304*(5676), 1494–1497. https://doi.org/10.1126/science.1096014

Berriri, S., Garcia, A. V., Dit Frey, N. F., Rozhon, W., Pateyron, S., Leonhardt, N., Montillet, J.-L., Leung, J., Hirt, H., & Colcombet, J. (2012). Constitutively Active Mitogen-Activated Protein Kinase Versions Reveal Functions of *Arabidopsis* MPK4 in Pathogen Defense Signaling. *The Plant Cell*, *24*(10), 4281–4293. https://doi.org/10.1105/tpc.112.101253

Bigeard, J., & Hirt, H. (2018). Nuclear Signaling of Plant MAPKs. *Frontiers in Plant Science*, *9*, 469. https://doi.org/10.3389/fpls.2018.00469

Camps, M., Nichols, A., & Arkinstall, S. (2000). Dual specificity phosphatases: A gene family for control of MAP kinase function. *The FASEB Journal*, *14*(1), 6–16. https://doi.org/10.1096/fasebj.14.1.6

Clough, S. J., & Bent, A. F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana: Floral dip transformation of Arabidopsis. *The Plant Journal*, *16*(6), 735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x

Colcombet, J., & Hirt, H. (2008). *Arabidopsis* MAPKs: A complex signalling network involved in multiple biological processes. *Biochemical Journal*, *413*(2), 217–226. https://doi.org/10.1042/BJ20080625

Danquah, A., De Zélicourt, A., Boudsocq, M., Neubauer, J., Frei Dit Frey, N., Leonhardt, N., Pateyron, S., Gwinner, F., Tamby, J.-P., Ortiz-Masia, D., Marcote, M. J., Hirt, H., & Colcombet, J. (2015). Identification and characterization of an ABA-activated MAP kinase cascade in *Arabidopsis thaliana*. *The Plant Journal*, *82*(2), 232–244. https://doi.org/10.1111/tpj.12808

Davis, R. J. (1993). The mitogen-activated protein kinase signal transduction pathway. *Journal of Biological Chemistry*, *268*(20), 14553–14556. https://doi.org/10.1016/S0021-9258(18)82362-6

Dreze, M., Monachello, D., Lurin, C., Cusick, M. E., Hill, D. E., Vidal, M., & Braun, P. (2010). High-Quality Binary Interactome Mapping. In *Methods in Enzymology* (Vol. 470, pp. 281–315). Elsevier. https://doi.org/10.1016/S0076-6879(10)70012-4

Farooq, A., Chaturvedi, G., Mujtaba, S., Plotnikova, O., Zeng, L., Dhalluin, C., Ashton, R., & Zhou, M.-M. (2001). Solution Structure of ERK2 Binding Domain of MAPK Phosphatase MKP-3. *Molecular Cell*, *7*(2), 387–399. https://doi.org/10.1016/S1097-2765(01)00186-1

Gaudet, P., Livstone, M. S., Lewis, S. E., & Thomas, P. D. (2011). Phylogenetic-based propagation of functional annotations within the Gene Ontology consortium. *Briefings in Bioinformatics*, *12*(5), 449–462. https://doi.org/10.1093/bib/bbr042

Guo, H., Feng, P., Chi, W., Sun, X., Xu, X., Li, Y., Ren, D., Lu, C., David Rochaix, J., Leister, D., & Zhang, L. (2016). Plastid-nucleus communication involves calcium-modulated MAPK signalling. *Nature Communications*, *7*(1), 12173. https://doi.org/10.1038/ncomms12173

Gupta, R., Huang, Y., Kieber, J., & Luan, S. (1998). Identification of a dual-specificity protein phosphatase that inactivates a MAP kinase from Arabidopsis. *The Plant Journal*, *16*(5), 581–589. https://doi.org/10.1046/j.1365-313x.1998.00327.x

Huang, Y., Li, H., Gupta, R., Morris, P. C., Luan, S., & Kieber, J. J. (2000). ATMPK4, an Arabidopsis Homolog of Mitogen-Activated Protein Kinase, Is Activated in Vitro by AtMEK1 through Threonine Phosphorylation. *Plant Physiology*, *122*(4), 1301–1310. https://doi.org/10.1104/pp.122.4.1301

Ichimura, K., Mizoguchi, T., Irie, K., Morris, P., Giraudat, J., Matsumoto, K., & Shinozaki, K. (1998). Isolation of ATMEKK1 (a MAP Kinase Kinase Kinase)-Interacting Proteins and Analysis of a MAP Kinase Cascade inArabidopsis. *Biochemical and Biophysical Research Communications*, *253*(2), 532–543. https://doi.org/10.1006/bbrc.1998.9796

Jagodzik, P., Tajdel-Zielinska, M., Ciesla, A., Marczak, M., & Ludwikow, A. (2018). Mitogen-Activated Protein Kinase Cascades in Plant Hormone Signaling. *Frontiers in Plant Science*, *9*, 1387. https://doi.org/10.3389/fpls.2018.01387

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. *Frontiers in Plant Science*, *9*, 1697. https://doi.org/10.3389/fpls.2018.01697

Kerk, D., Bulgrien, J., Smith, D. W., Barsam, B., Veretnik, S., & Gribskov, M. (2002). The Complement of Protein Phosphatase Catalytic Subunits Encoded in the Genome of Arabidopsis. *Plant Physiology*, *129*(2), 908–925. https://doi.org/10.1104/pp.004002

Kerk, D., Templeton, G., & Moorhead, G. B. G. (2008). Evolutionary Radiation Pattern of Novel Protein Phosphatases Revealed by Analysis of Protein Data from the Completely Sequenced Genomes of Humans, Green Algae, and Higher Plants. *Plant Physiology*, *146*(2), 323–324. https://doi.org/10.1104/pp.107.111393

Kosetsu, K., Matsunaga, S., Nakagami, H., Colcombet, J., Sasabe, M., Soyano, T., Takahashi, Y., Hirt, H., & Machida, Y. (2010). The MAP Kinase MPK4 Is Required for Cytokinesis in *Arabidopsis thaliana*. *The Plant Cell*, *22*(11), 3778–3790. https://doi.org/10.1105/tpc.110.077164

Krysan, P. J., & Colcombet, J. (2018). Cellular Complexity in MAPK Signaling in Plants: Questions and Emerging Tools to Answer Them. *Frontiers in Plant Science*, *9*, 1674. https://doi.org/10.3389/fpls.2018.01674

Liang, J., Zhang, Q., Liu, Y., Zhang, J., Wang, W., & Zhang, Z. (2022). Chlorosis seedling lethality 1 encoding a MAP3K protein is essential for chloroplast development in rice. *BMC Plant Biology*, *22*(1), 20. https://doi.org/10.1186/s12870-021-03404-9

Lichtenthaler, H. K., & Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochemical Society Transactions*, *11*(5), 591–592. https://doi.org/10.1042/bst0110591

Li, L., Nelson, C. J., Trösch, J., Castleden, I., Huang, S., & Millar, A. H. (2017). Protein Degradation Rate in *Arabidopsis thaliana* Leaf Growth and Development. *The Plant Cell*, *29*(2), 207–228. https://doi.org/10.1105/tpc.16.00768

Lin, C., & Chen, S. (2018). New functions of an old kinase MPK4 in guard cells. *Plant Signaling & Behavior*, *13*(5), e1477908. https://doi.org/10.1080/15592324.2018.1477908

Liu, Y., Shepherd, E. G., & Nelin, L. D. (2007). MAPK phosphatases—Regulating the immune response. *Nature Reviews Immunology*, *7*(3), 202–212. https://doi.org/10.1038/nri2035

Liu, R., Xu, Y.-H., Jiang, S.-C., Lu, K., Lu, Y.-F., Feng, X.-J., Wu, Z., Liang, S., Yu, Y.-T., Wang, X.-F., & 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. *Journal of Experimental Botany*, *64*(18), 5443–5456. https://doi.org/10.1093/jxb/ert307

Liu, R., Liu, Y., Ye, N., Zhu, G., Chen, M., Jia, L., Xia, Y., Shi, L., Jia, W., & Zhang, J. (2015). AtDsPTP1 acts as a negative regulator in osmotic stress signalling during Arabidopsis seed germination and seedling establishment. *Journal of Experimental Botany*, *66*(5), 1339–1353. https://doi.org/10.1093/jxb/eru484

Lee, J. S., & Ellis, B. E. (2007). Arabidopsis MAPK Phosphatase 2 (MKP2) Positively Regulates Oxidative Stress Tolerance and Inactivates the MPK3 and MPK6 MAPKs. *Journal of Biological Chemistry*, *282*(34), 25020–25029. https://doi.org/10.1074/jbc.M701888200

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. *The Plant Journal*, *57*(6), 975–985. https://doi.org/10.1111/j.1365-313X.2008.03741.x

Lumbreras, V., Vilela, B., Irar, S., Solé, M., Capellades, M., Valls, M., Coca, M., & Pagès, M. (2010). MAPK phosphatase MKP2 mediates disease responses in Arabidopsis and functionally interacts with MPK3 and MPK6: MKP2 mediates disease responses and interacts with MPK6 and MPK3. *The Plant Journal*, *63*(6), 1017–1030. https://doi.org/10.1111/j.1365-313X.2010.04297.x

Marmagne, A., Ferro, M., Meinnel, T., Bruley, C., Kuhn, L., Garin, J., Barbier-Brygoo, H., & Ephritikhine, G. (2007). A High Content in Lipid-modified Peripheral Proteins and Integral Receptor Kinases Features in the Arabidopsis Plasma Membrane Proteome. *Molecular & Cellular Proteomics*, *6*(11), 1980–1996. https://doi.org/10.1074/mcp.M700099-MCP200

Marshall, C. J. (1994). MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. *Current Opinion in Genetics & Development*, *4*(1), 82–89. https://doi.org/10.1016/0959-437X(94)90095-7

Monroe-Augustus, M., Zolman, B. K., & Bartel, B. (2003). IBR5, a Dual-Specificity Phosphatase-Like Protein Modulating Auxin and Abscisic Acid Responsiveness in Arabidopsis. *The Plant Cell*, *15*(12), 2979–2991. https://doi.org/10.1105/tpc.017046

Naoi, K., & Hashimoto, T. (2004). A Semidominant Mutation in an Arabidopsis Mitogen-Activated Protein Kinase Phosphatase-Like Gene Compromises Cortical Microtubule Organization[W]. *The Plant Cell*, *16*(7), 1841–1853. https://doi.org/10.1105/tpc.021865

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., & Mundy, J. (2000). Arabidopsis MAP Kinase 4 Negatively Regulates Systemic Acquired Resistance. *Cell*, *103*(7), 1111–1120. https://doi.org/10.1016/S0092-8674(00)00213-0

Pogson, B. J., & Albrecht, V. (2011). Genetic Dissection of Chloroplast Biogenesis and Development: An Overview. *Plant Physiology*, *155*(4), 1545–1551. https://doi.org/10.1104/pp.110.170365

Pogson, B. J., Ganguly, D., & Albrecht-Borth, V. (2015). Insights into chloroplast biogenesis and development. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1847*(9), 1017–1024. https://doi.org/10.1016/j.bbabio.2015.02.003

Pytela, J., Kato, T., & Hashimoto, T. (2010). Mitogen-activated protein kinase phosphatase PHS1 is retained in the cytoplasm by nuclear extrusion signal-dependent and independent mechanisms. *Planta*, *231*(6), 1311–1322. https://doi.org/10.1007/s00425-010-1135-8

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*: PHS1 *gene is involved in abscisic acid signalling*. *The Plant Journal*, *47*(5), 711–719. https://doi.org/10.1111/j.1365-313X.2006.02823.x

Raines, C. A. (2003). The Calvin cycle revisited. *Photosynthesis Research*, *75*(1), 1–10. https://doi.org/10.1023/A:1022421515027

Ramazi, S., & Zahiri, J. (2021). Post-translational modifications in proteins: Resources, tools and prediction methods. *Database*, *2021*, baab012. https://doi.org/10.1093/database/baab012

Rodriguez, M. C. S., Petersen, M., & Mundy, J. (2010). Mitogen-Activated Protein Kinase Signaling in Plants. *Annual Review of Plant Biology*, *61*(1), 621–649. https://doi.org/10.1146/annurev-arplant-042809-112252

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D., & Lohmann, J. U. (2005). A gene expression map of Arabidopsis thaliana development. *Nature Genetics*, *37*(5), 501–506. https://doi.org/10.1038/ng1543

Shi, H., Li, Q., Luo, M., Yan, H., Xie, B., Li, X., Zhong, G., Chen, D., & Tang, D. (2022). BRASSINOSTEROID-SIGNALING KINASE1 modulates MAP KINASE15 phosphorylation to confer powdery mildew resistance in Arabidopsis. *The Plant Cell*, *34*(5), 1768–1783. https://doi.org/10.1093/plcell/koac027

Silva, L. A. S., Sampaio, V. F., Barbosa, L. C. S., Machado, M., Flores-Borges, D. N. A., Sales, J. F., Oliveira, D. C., Mayer, J. L. S., Kuster, V. C., & Rocha, D. I. (2020). Albinism in plants – far beyond the loss of chlorophyll: Structural and physiological aspects of wild-type and albino royal poinciana (*Delonix regia*) seedlings. *Plant Biology*, *22*(5), 761–768. https://doi.org/10.1111/plb.13146

Tamnanloo, F., Damen, H., Jangra, R., & Lee, J. S. (2018). MAP KINASE PHOSPHATASE1 Controls Cell Fate Transition during Stomatal Development. *Plant Physiology*, *178*(1), 247–257. https://doi.org/10.1104/pp.18.00475

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. https://doi.org/10.1007/s00425-015-2447-5

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*(1), 141–152. https://doi.org/10.1016/j.molcel.2004.06.023

Teramoto, H., Ono, T., & Minagawa, J. (2001). Identification of Lhcb Gene Family Encoding the Light-harvesting Chlorophyll-a/b Proteins of Photosystem II in Chlamydomonas reinhardtii. *Plant and Cell Physiology*, *42*(8), 849–856. https://doi.org/10.1093/pcp/pce115

Ulm, R. (2002). Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. *The EMBO Journal*, *21*(23), 6483–6493. https://doi.org/10.1093/emboj/cdf646

Walia, A., Lee, J. S., Wasteneys, G., & Ellis, B. (2009). Arabidopsis mitogen-activated protein kinase MPK18 mediates cortical microtubule functions in plant cells. *The Plant Journal*, *59*(4), 565–575. https://doi.org/10.1111/j.1365-313X.2009.03895.x

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*. *The Plant Cell*, *19*(1), 63–73. https://doi.org/10.1105/tpc.106.048298

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., & Provart, N. J. (2007). An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets. *PLoS ONE*, *2*(8), e718. https://doi.org/10.1371/journal.pone.0000718

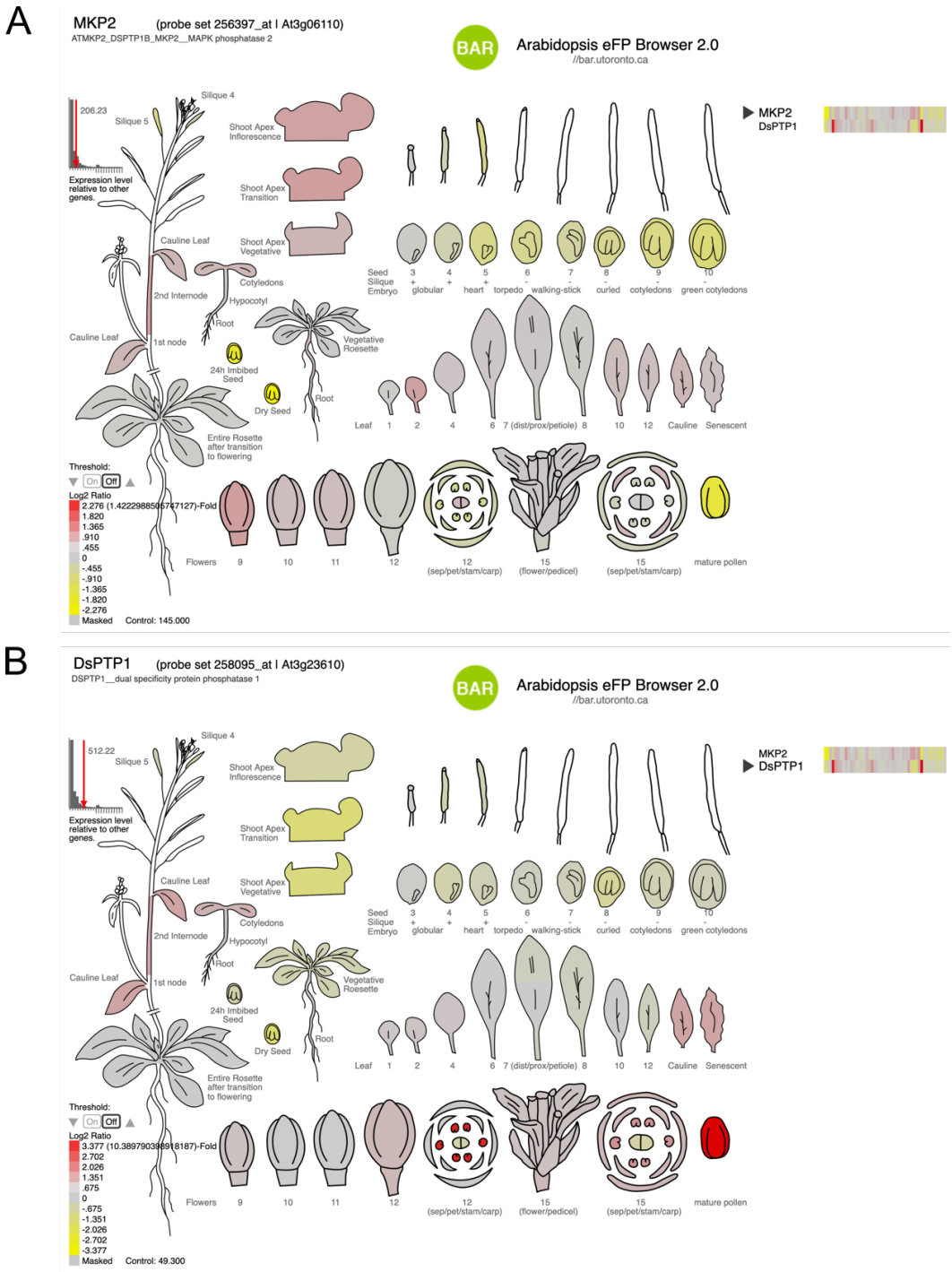
Xu, J., Xie, J., Yan, C., Zou, X., Ren, D., & Zhang, S. (2014). A chemical genetic approach demonstrates that MPK3/MPK6 activation and NADPH oxidase-mediated oxidative burst are two independent signaling events in plant immunity. *The Plant Journal*, *77*(2), 222–234. https://doi.org/10.1111/tpj.12382

Zhang, W., Cochet, F., Ponnaiah, M., Lebreton, S., Matheron, L., Pionneau, C., Boudsocq, M., Resentini, F., Huguet, S., Blázquez, M. Á., Bailly, C., Puyaubert, J., & Baudouin, E. (2019). The MPK 8- TCP 14 pathway promotes seed germination in Arabidopsis. *The Plant Journal*, *100*(4), 677–692. https://doi.org/10.1111/tpj.14461

Zhang, X., Henriques, R., Lin, S.-S., Niu, Q.-W., & Chua, N.-H. (2006). Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. *Nature Protocols*, *1*(2), 641–646. https://doi.org/10.1038/nprot.2006.97

Zhao, B. S., Roundtree, I. A., & He, C. (2017). Post-transcriptional gene regulation by mRNA modifications. *Nature Reviews Molecular Cell Biology*, *18*(1), 31–42. https://doi.org/10.1038/nrm.2016.132

Appendix



Supplementary Figure S1. Relative tissue-specific expression values of (A) *MKP2* and (B) *DsPTP1* in *Arabidopsis thaliana*. The outputs were obtained from the eFP Browser 2.0 and are accessible on the BAR webserver (http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFPBrowser2.html). The data is derived from Winter et al., 2007.