Identification of a Mitogen Activated Protein Kinase Phosphatase Controlling Stomatal Development in *Arabidopsis thaliana*

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A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science at

Concordia University

Montreal, Quebec, Canada

January 2018

CONCORDIA UNIVERSITY
School of Graduate Studies

This is to certify that the thesis prepared

By: Hassan Damen

Entitled: Identification of a Mitogen Activated Protein Kinase Phosphatase Controlling Stomatal Development in *Arabidopsis thaliana*

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

Master of Sciences (Biology)

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Abstract

Identification of a Mitogen Activated Protein Kinase Phosphatase Controlling Stomatal Development in *Arabidopsis thaliana*

Hassan Damen, M.Sc.

Stomata are tiny openings on all above ground plant epidermis except for petals and stamen filaments. They regulate gas exchange and control water loss, which enables plants to adapt to environmental changes and facilitates photosynthesis. In *Arabidopsis*, stomata are formed through a series of differentiation events according to positional cues mediated by cell-to-cell signalling that contains mitogen-activated protein kinase (MAPK) cascade components, an YDA (MAPKKK), MKK4/MKK5 (MAPKKs), MPK3/MPK6 (MAPKs). Although much is known about the upstream kinases and biological role of MAPKs (MPK3 and MPK6) during stomatal development, the process by which they are inactivated remains unclear. In mammals, some members of the dual-specificity phosphatases (DSPs), which dephosphorylate both phosphoserine/threonine and phosphotyrosine residues of their substrates, have been recognized as key players in deactivating MAPKs. Five DSP-type phosphatases with described or predicted MAPK phosphatase (MKP) function are encoded in the genome of *Arabidopsis*, but their role in stomatal patterning and differentiation has not yet been investigated.

Here, I report the identification of MKP1, one of the five *Arabidopsis* MKPs, as a key regulator promoting stomatal cell fate differentiation. A loss-of-function *mkp1* mutation occasionally developed clusters of small cells that failed to differentiate into stomata. I determined the phosphatase activity of MKP1 is essential and required for totally normal stomatal development. A genetic analysis showed that the redundantly functioning kinases, *MPK3* and *MPK6*, are epistatic to *MKP1,* consistent with the predicted function of MKP1 as a MAPK phosphatase. Furthermore, our cell type-specific rescue experiments using the promoters of the genes encoding the stomatal basic-helix-loop-helix (bHLH) transcription factors revealed that early stomatal cell-specific expression of MKP1 is sufficient for proper stomatal development. This data implies that MKP1 controls a MAPK cascade in the first out of three division and/or differentiation “steps” of stomatal development. Taken together, these findings help determine

the role of phosphatase in controlling MAPK signaling specificity in stomatal development, and thus providing further insights into the cell fate regulation by this intracellular signalling cascade.

Acknowledgements

I would like to thank my supervisor Dr. Lee, for her guidance and great help that have made this work possible. I would also like to thank my committee members Dr. Gulick and Dr. Whiteway for their guidance and advice

I would like to thank my awesome wife Roula El Maoula, for her patience during my pursuit of this degree.

I would like to dedicate this work to the mighty martyrs of my country, who with their blood wrote the greatest signs of sacrifice and loyalty.

Finally, I would also like to thank my labmates: Dr. Longgang Cui, Farzaneh Tamnanloo, Daniel Gaudet, and lab assistant Leila Altken for their great help.

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List of Abbreviations

ABA	Abscisic acid
bHLH	Basic helix-loop-helix
CA	Constitutively active
cDNA	Complementary DNA
Col	Colombia
D	Aspartic acid
DNA	Deoxyribonucleic acid
DsPTP	Dual-specificity protein tyrosine phosphatase
E	Glutamic acid
ERf	Erecta receptor family
FLC	Flowering locus
GC	Guard cell
GFP	Green fluorescent protein
GH	Gelsolin homology
GMC	Guard mother cell
GUS	β-glucuro nidase
HYG	Hygromycin
IBR	Indole 3-Butyric acid response
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MKP	Mitogen-activated protein kinase phosphatase

MMC	Meristemoid mother cell
M	Meristemoid
GMC	Guard mother cell
mRNA	Messenger RNA
MS	Murashige and Skoog
P	Proline
PC	Pavement cells
PCR	Polymerase chain reaction
PHS	Propizymide hypersensitive
PP2C	Type 2C protein phosphatases
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
RNAi	RNA interference
S	Serine
SA	Salicylic acid
SID2	Salicylic acid induction deficient 2
SIPK	Salicylic Acid-induced Protein Kinase
SLGC	Stomata lineage ground cell
SNC1	SUPPRESSOR OF npr1-1, CONSTITUTIVE 1
T	Threonine
TBO	Toluidine blue
T-DNA	Transfer DNA
Ws	Wassilewskija

WT	Wild type
X	Any amino acid
Y	Tyrosine
Y2H	Yeast two hybrid
YFP	Yellow fluorescent protein

1. Introduction

1.1. Mitogen-Activated Protein Kinase (MAPK) signaling

Like all other living organisms, plants must constantly respond and adapt to changes in the external environment in order to sustain normal growth and development. This relies on perceiving cues from the environment and activating signaling machinery to regulate the appropriate physiological and genetic responses. Many different research groups have studied the signaling molecules and pathways involved, and these studies have revealed that protein phosphorylation, catalyzed by protein kinases, is one of the major mechanisms for controlling cellular functions. Several different types of protein kinases have been identified from plants but these all belong to the protein kinase super-family (Lehti-Shiu and Shiu, 2012). One of the most important kinase groups involved in plant signal transduction pathways consists of the members of the mitogen-activated protein kinase (MAPK) cascades (Nakagami *et al*., 2005).

MAPK signalling cascades consist of three interlinked protein kinases, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK, that are sequentially activated to trigger specific output responses, such as changes in gene expression, differentiation, development, and stress responses (Hamel *et al*., 2006). In response to environmental conditions and developmental cues, MAKKKs can be activated and therefore generate a downstream signal by phosphorylating the conserved serine/threonine residues in a conserved -S/T-X_{3-5}-S/T- motif of MAPKKs. Phosphorylation signal relays from MAPKKs to specific threonine and tyrosine residues in the -TXY- motif of the MAPK catalytic domain (Widmann *et al*., 1999). It has been shown that the phosphorylation event of both threonine and tyrosine in this motif is required for full activation of the MAPK (Widmann *et al*., 1999). The activated MAPK, as the terminal component of this sequential cascade, acts as a serine threonine kinase that is able to phosphorylate a wide range of cellular targets such as transcription factors, cytoskeleton proteins, protein phosphatases and other protein kinases. This phosphorylation could occur on serine and/or threonine residues in its substrate at a consensus motif PXT/SP. (Masuda *et al*., 2001; Feilner *et al*., 2005).

Considering the diverse functions that MAPKs play, the organization of the MAPK signalling components into functional signalling modules by scaffold proteins have been suggested critical for this specificity in signal transduction. It is not clear how this specificity is accomplished but

some studies have revealed the selective motifs that control the strength and orientation of physical interactions between the kinase and its substrate, resulting in the prevention of the pathway components from any unsuitable cross-talk (Morrison and Davis, 2003). In addition to the specificity of MAPK for their substrate(s), the magnitude and duration of kinase activation are crucial in determining the physiological outcome of protein kinase-based signaling (Marshall**,** 1995).

More MAPK genes found within the plant genome compared to any other organism which suggested the involvement of these kinases in several advanced processes in cell signaling. (Meskiene and Hirt, 2000). Indeed, plant MAPK signaling has been demonstrated to be involved in responses to a wide range of physiological, developmental and hormonal processes and its activation has been correlated with stimulatory treatments such as wounding, pathogen infection, temperature, touch, hormones, osmotic stress and reactive oxygen species (Tena *et al*., 2001; Zhang *et al*., 2001; Nakagami *et al*., 2005). Arabidopsis has 20 MAPKs, 10 MKKs and around 60 MAPKKKs, allowing for a great number of combinational interactions (MAP Kinase Group, 2002). So far, however, only a limited number of interactions have been described and these show that MAPK cascade elements are promiscuous among different signaling transduction pathways, a commonality with yeast and mammalian counterparts (Andreasson and Ellis, 2010). When compared phylogenetically with mammalian MAPKs, all plant MAPKs belong to a single group, the so-called ERK-type sub-family (Tena *et al.,* 2001). The canonical members of this family (ERK1 and ERK2) are often activated in response to growth factors in mammals, but in plants, MAPKs have been shown to be activated by a much broader range of stimuli. Based on phylogenetic analysis, plant MAPKs can be further clustered into four distinct groups, A-D (Hamel *et al*., 2006). Group A MAPKs include the two most extensively studied MAPKs, MPK3 and MPK6. These two MAPKs have been found to be involved in numerous processes including environmental, hormonal, and developmental responses like stomatal patterning and differentiation (Droillard *et al*., 2000; Liu *et al*., 2003; Miles *et al*., 2005; Wang *et al*., 2007). With respect to group B, some of its members found to be involved in cell division like MPK13 and others in environmental stress responses like MPK4 but the majority is still not well characterized (Calderini *et al.,* 1998; Bögre, 1999; Droillard *et al.*, 2004). Group C has limited functional information whereas, group D MAPKs with their TDY instead of TEY, and have been studied in several plant species. (Meskiene and Hirt, 2000; Nakagami *et al*., 2005).

1.2 MAPK phosphatases (MKPs)

The balance between activation by upstream MAKKs and inactivation by protein phosphatases considered as an optimal regulator for the activity of MAPK (Pearson *et al*., 2001). Phosphorylation of both the serine/threonine and tyrosine of the (TXY) motif converts MAPK into an active form. Therefore, dephosphorylation by phosphatase protein of those residues could be the statue of reconversion into an inactive form (Camps *et al*., 2000).

Phosphatases are classified into two classes that could downregulate MAPKs: Protein-Tyrosine Phosphatases (PTPs) and protein-serine/threonine phosphatases. Dual-specificity protein phosphatases (DsPTPs), a subclass of the PTPs, are known to dephosphorylate both serine/threonine and tyrosine residues in their substrates. Number of DsPTPs have been identified as MAPK phosphatases (MKPs) in the mammalian systems.

The mammalian MKPs that have been characterized are known to have a common animo-acid sequence with high levels of identity within the catalytic domains. This rapprochement including the conserved phosphatase active-site motif (Theodosiou and Ashworth, 2002). Arabidopsis genome is encoding for around 23 dual-specificity protein phosphatases, but only five of them have the MKP-unique AY[L/I]M motif found in all mammalian MKPs. Those five are MAP kinase phosphatase 1 (MKP1), MAPK phosphatase 2 (MKP2), The INDOLE-3-BUTYRIC ACID RESPONSE5 (IBR5), PROPYZAMIDE-HYPERSENSITIVE 1 (PHS1), and dual specificity protein phosphatase1 (DsPTP1) (Kerk *et al*., 2002). This contrast between large families of MAPKs and the small number of MKPs in plants suggest that individual MKPs may regulate several MAPKs to provide crucial roles in the intracellular signaling.

Among these five Arabidopsis MKPs, MKP1 found to be expressed widely in various plant tissues. It has been identified during various environmental stresses as a crucial regulator of the MAPK pathways (Ulm, *et al*., 2001; Ulm *et al*., 2002; Bartels *et al*., 2009)

The *mkp1* null mutant was initially identified in the *Arabidopsis Wassilewskija* (Ws) with no aberrant morphological phenotype (Ulm *et al*., 2001). Interestingly, the introduction of this mutation into the *Arabidopsis Columbia* genome resulted in a dwarf growth phenotype with aberrant leaf development, long pedicels, misshapen inflorescences, early senescence and reduced fertility (Ulm *et al*., 2001). These aberrant *mkp1*(Col) phenotypes are caused by the

increased salicylic acid (SA) accumulation. A loss-of-function mutation of the resistance gene-like *SUPPRESSOR OF npr1-1*, *CONSTITUTIVE 1* (*SNC1*) rescue the *mkp1* (Col) growth phenotype. This gene does not exist in *Arabidopsis* (Ws) thus explaining the absence of the aberrant growth phenotype of the *mkp1* null mutant in Ws (Bartels *et al.,* 2009). Null *mkp1* mutant shows hypersensitivity to genotoxic stress and UV light with more resistance to elevated salinity (Ulm *et al.,* 2002). *In vitro* yeast two-hybrid (*Y2H*) assays confirmed the interaction between *Arabidopsis* MKP1 and the stress-activated MAPKs MPK3, MPK4 and MPK6. Of these, the interaction between MKP1 and MPK6 is most pronounced (Ulm *et al.,* 2002). Expression pattern study using YFP-MKP1 recombinant protein revealed that the main localization of the MKP1 protein is concentrated in the cytoplasm, where it interacts with MPK6 (Bartels *et al.,* 2009). At the same time, a recent study demonstrated MKP1 as a protein targeted to both cytosol and peroxisomes (Kataya *et al.,* 2015). Also *mkp1*(Col) mutant shows resistance to infection from bacterial pathogen *Pseudomonas syringae* due to the enhanced activity of MPK6 (Bartels *et al.,* 2009).

Another tyrosine phosphatase called Protein Tyrosine Phosphatase1 (PTP1) shows partially overlapping functions with MKP1 in repressing SA synthesis. *mkp1*(Col) and *mkp1ptp1* double mutants show elevated levels of SA, which is probably caused by increased activity of MPK3 and MPK6. Mutation of these two kinases have been found to suppress *mkp1* phenotypes, which indicate that MKP1 and PTP1 negatively regulate MPK3/6 during the SA-dependent defence responses (Bartels *et al*., 2009). As mentioned before, *mkp1* mutant induced hyper accumulation of SA which could be suppressed by a SA–degrading hydroxylase (NahG) from *Pseudomonas* bacteria (Suzuki *et al.,* 2000). *mkp1NahG* transgenic plant shows normal growth compared to *mkp1* mutant plant which again confirms the aberrant phenotype relativity to SA accumulation. Another gene found in *Arabidopsis*, called salicylic acid induction deficient 2 (*SID2*), was found to be involved in SA biosynthesis. *sid2* mutant interrupts SA synthesis and causes an increase in seed yield (Abreu and Munné-Bosch , 2009). MKP1 also directly interacts with a Ca^{2+} binding proteins, calmodulins, through the CaM-binding domain. This interaction is important for the dephosphorylation activity of MKP1 (Yamakawa *et al.,* 2004; Kyunghee Lee *et al.,* 2008). Another evidence proved that catalytic activation of the tobacco *NtMKP1* is controlled by the physiological substrate SA-induced protein kinase (SIPK). SIPK were shown to be

dephosphorylated by MKP1, which lead to the repression of SA biosynthesis (Katou *et al*., 2005. Bartels *et al.,* 2009).

MKP2 found to be involved in oxidative stress tolerance. Suppression of MKP2 during ozone treatment prolonged the activity of MPK3 and MPK6, however, MKP2 overexpression led to a dephosphorylation of these kinases. This results provided a strong suggestion that MPK3 and MPK6 could be a direct target for MKP2 which considered as a positive regulator of plant response against oxidative stress (Lee and Ellis, 2007). On the other hand, and at the level of plant immunity, MKP2 has shown to have a crucial role during pathogen defence responses. *mkp2* knockout increased the strength of plant immunity against bio trophic pathogens, while this mutation had a counter result during necrotrophic pathogen attack. This fundamental pathogen response of MKP2 is exhibited by regulating both of MPK3 and MPK6 through a direct interaction that results with an inactivated kinases via dephosphorylation. (Lumbreras *et al*., 2010).

IBR5 is reported as a major phosphatase involved in auxin signalling through its physical interaction and dephosphorylation of MPK12 (Lee *et al*., 2008). Knockout mutant of *IBR5* gene reduced plant sensitivity to auxin. This result demonstrated that IBR5 is a positive regulator of auxin signalling (Lee *et al*., 2008). Moreover, IBR5 found to play an important role during low temperature condition. It interacts with CHS3 (chilling-sensitive) protein where this interaction is mediated by the phosphatase activity of IBR5, so resulted in protection of CHS3. Knockout mutation *ibr5* showed an inhibition of CHS3 accumulation during low and chilling temperature in Arabidopsis (Liu *et al*., 2015).

PHS1 is involved in microtubule formation, which is mediated through its interaction with MPK18 (Walia *et al*., 2009). *mpk18* mutant plants exhibited malfunction of root microtubules, a phenotype which disappeared when PHS1 is mutated. These results demonstrated that PHS1 is a negative regulator of cortical microtubule function in the plant roots (Walia *et al*., 2009). Recently, PHS1 found to be a positive regulator of plant flowering. Knock out *phs1* showed high expression of FLOWERING LOCUS C (FLC) while inhibition of CONSTANS (CO) and FLOWERING LOCUS T (FT) expression. These results demonstrated that PHS1 is a direct modulator of CO and FLC levels during plant flowering (Tang *et al.*, 2016).

DsPTP1 regulates abscisic acid (ABA) accumulation and affects seed germination due to its involvement in osmotic stress signalling as a negative regulator (Liu *et al.,* 2015).Under osmotic stress dsptp1 knockout mutant plants revealed high seed germination with enhanced root elongation, a phenotype is reversed in overexpression line of DsPTP1.

Serine-Threonine protein phophatases (PP2C) can dephosphorylate the substrate at a conserved Thr-X-Tyr motif, where X is any amino acid. MP2C (*Medicago sativa*) has been discovered as the first plant PP2C that interacts and deactivates MAPKs (Meskiene, *et al.,* 2003). It is involved in defense responses during mechanical injury (Baudouin *et al.,* 1999). AP2C1 is another PP2C involved in wounding reaction through its interaction with MPK4 and MPK6. AP2C1 was also found to be involved in innate immunity against fungal infection (Baudouin *et al.,* 1999). It is noteworthy that the *Arabidopsis* AP2C3 protein, which belongs to the PP2C family, was characterized as a critical player in stomata development. It interacts with MPK3, MPK4 and MPK6 and is localized within the nucleus. Recent study shows that overexpression of *AP2C3* downregulates *MKP3/MKP6*, and thus creating an excess entry into stomata lineage cells (Umbrasaite *et al*., 2010).

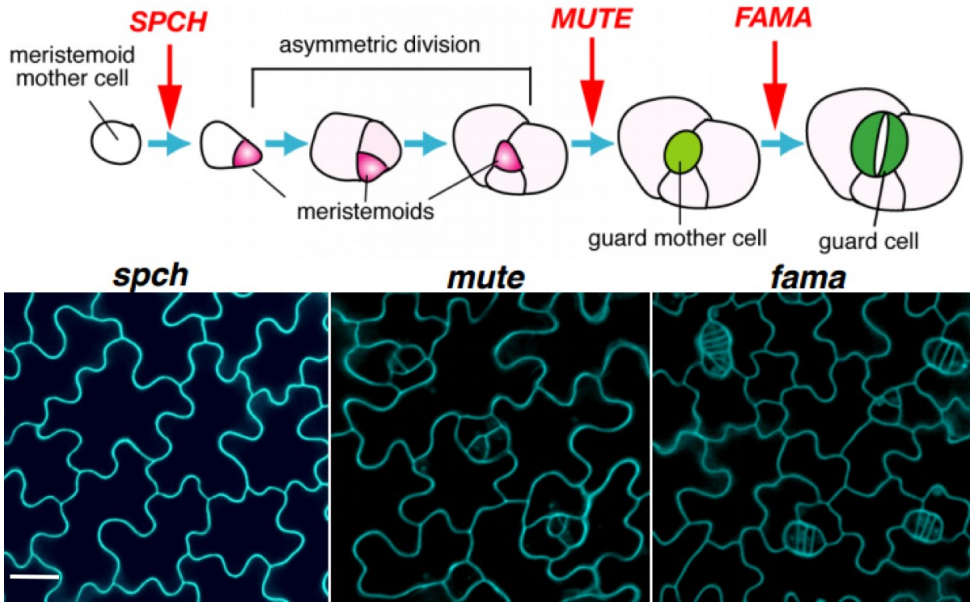
Protein Tyrosine phosphatases (PTP) is involved in many processes, mainly in development and abiotic/biotic stress responses. To date, it is not well understood how PTPs act, except for one member called PTP1. Environmental stresses are found to alter the expression of *PTP1* gene due to its involvement in a negative feedback loop that controls MAPK activity (Xu *et al.,* 1998). As mentioned previously that PTP1 negatively regulates MPK3/6 during the SA-dependent defence responses (Bartels *et al.,* 2009).

1.3 Stomatal Development

Stomatal development has recently emerged as an excellent model to study cell-to-cell signaling due to its simple cell types, sensitivity, and accessibility in plants. Stomata are the respiratory organs in plants, prevalent on all above ground epidermis aiding the preparation of nutrients for survival through photosynthesis and for regulation of transpiration (Schroeder *et al.,* 2001). In Arabidopsis, stomatal development begins as a subset of protodermal cells, meristemoid mother cells (MMCs), which undergo asymmetric cell division that creates a stomatal precursor called a meristemoid. The meristemoid reiterates asymmetric cell divisions and produces surrounding non-stomatal cells (stomatal lineage ground cells; SLGCs) before differentiating into the next precursor, a guard mother cell (GMC). The GMC undergoes a single symmetric division to terminally differentiate into paired guard cells that constitute a stoma (Figure 1). Major transitions in stomatal development in Arabidopsis are controlled by the sequential expression of three closely related basic-helix-loop-helix (bHLH) transcription factors, SPEECHLESS (SPCH), MUTE, and FAMA (Gudesblat *et al*., 2012; Alister *et al*., 2007; Pillitteri *et al*., 2007; Bhave *et al*., 2009; Ohashi-Ito and Bergmann, 2006). SPCH controls entry into asymmetric cell divisions, MUTE differentiation from meristemoid to guard mother cell, and FAMA differentiation from GMC to guard cell. Consequently, the loss of function *spch* results in an epidermis composed of only non-stomatal lineage puzzle-shaped pavement cells (MacAlister *et al*., 2007). The *mute* mutation also results in a stomataless phenotype except the stomatal lineage is arrested in the meristemoid cell stage (Pillitteri *et al*., 2007), and *fama* gives a phenotype of aberrantly divided guard mother cells that fail to differentiate into guard cells (Ohashi-Ito and Bergmann 2006). These stage-specific bHLH proteins are joined by a pair of more distantly related bHLHs, SCREAM (SCRM)/ICE1 and SCRM2, that redundantly affect all transitional stages of stomatal development (Kanaoka *et al.,* 2008). The density and distribution of stomata in leaves is highly plastic and emerges from interplay between the genetic and environmental factors experienced by the growing plants. As such, cell-to-cell communication specifying tissue patterning is very important for proper stomatal development in plants. This cell spacing is achieved by intercellular signalling, starting with an activation of transmembrane receptors ((Too Many Mouth) (TMM) and three ERECTA-family, ERECTA, ERL1, and ERL2) by its peptide ligands Epidermal Patterning Factor (EPFs), in response to environmental or developmental

conditions. This activation generates a downstream signal through the MAPK signaling components, resulting in the stimulation of pavement cell differentiation and inhibition of stomatal initiation, likely through suppressing the activity of bHLH transcription factors specifying stomatal differentiation (Wang *et al*., 2007; Lee *et al*., 2015).

Figure 1.



(Kanaoka et al., 2008)

Figure 1. Stomatal Development and the *bHLH* transcription factors

Stomatal development starts from a MMC that differentiates into M in the presence of SPCH. Asymmetric division occurs multiple times until the expression of MUTE that control the transition state of M into GMC. Symmetric division happens in the presence of FAMA that control the last stage of development where GMC becomes a guard cell. Lower images showing defects of the epidermis after the mutation of each stomatal transcription factor.

1.4 MAPK signaling in stomatal development

In *Arabidopsis*, stomatal development is regulated by MAPK-dependent processes. Discovery of the involvement of the MAPK cascade in stomatal differentiation came from the characterization of *yda* (*YDA*, MAPKKK) mutants, which resulted in the overproliferation of stomata in the epidermis, and native promoter driven expression of constitutively active YDA (CA-YDA) eliminates stomata (Bergmann *et al.,* 2004). Downstream of YDA, MKK4/5/7/9 (MAPKKs) and MPK3/6 (MAPKs) were then linked to this pathway (Colcombet and Hirt, 2008). Specific roles of these MAPK components in three discrete stages of stomatal development were investigated by expressing constitutively active and/or dominant negative forms of YDA and all *Arabidopsis* MKKs under the control of stomatal cell-type specific promoters (Lampard *et al*., 2009). MAPK components (YDA, MKK4/5/7/9, and MPK3/6,) inhibit stomatal initiation and M transition to GMC in first two stages of stomatal development. However, when constitutively active versions of these MAPK components were expressed with the last step of stomatal linage-specific stomatal promoter, YDA and MKK7/9, but notably not MKK4/5 and MPK3/6, induced the production of stomatal clusters, indicating their potential role in promoting GMC transition to GC, while inhibiting stomatal formation in earlier two steps of stomatal linage (Pillitteri *et al*., 2013). Phosphorylation of SPCH by MPK3/6 could takes place within its target domain (MPKTD) which leads to the suppression of SPCH activity (Lampard *et al.,* 2008). Among the stomatal bHLH proteins, a MPKTD is only found in SPCH and there is no reported evidence of MAPK regulation of other bHLHs, MUTE and FAMA, nor of the mechanism of SPCH downregulation.

Figure 2.

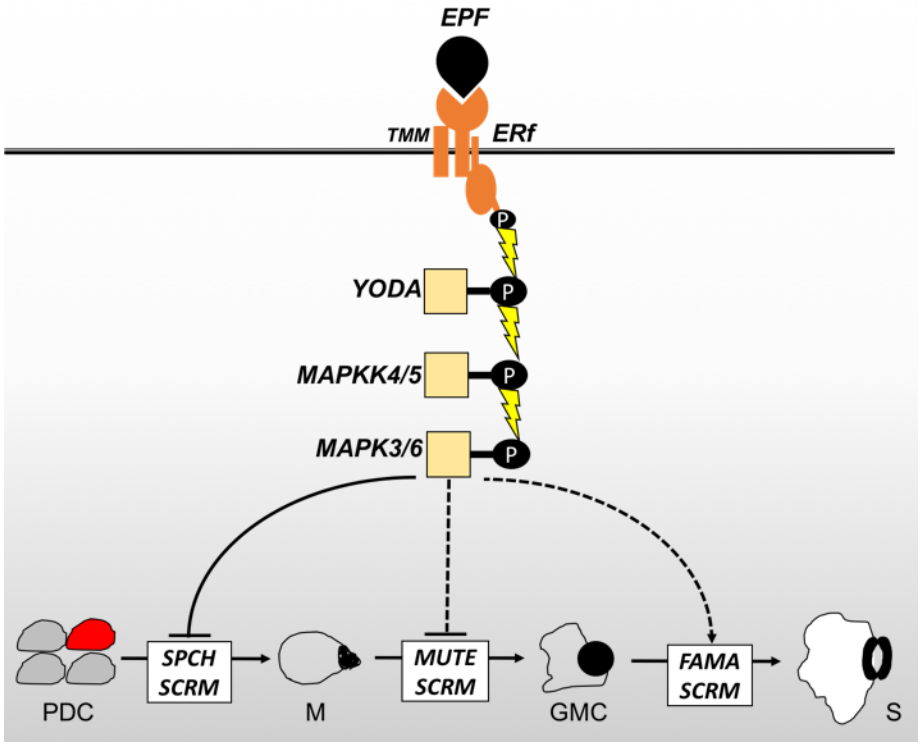


Figure 2. Signaling pathway and its component that regulate stomatal development.

EPF ligands generate signalling via their interaction with TMM and the ERf receptors. Signal translated to YDA, MKKs then MPKs. MPK3/6 phosphorylate SPCH inactivating it to reduce the initiation division. Predicted effects on MUTE and FAMA are labelled with dashed lines

1.5 Objective

How long a MAPK, an important final determinant of biological response, remains active can be affected either by how long the upstream MAPKKs remain active and/or whether a MAPK phosphatase activity is induced. Although much is known about the potential upstream stomatal kinases (YDA and MKK4/MKK5) and biological role of MPK3 and MPK6 during stomatal development, the process by which they are inactivated through the action of phosphatases such as MKPs remains unclear. Thus, the central goal of my thesis was to study the role of MKPs in controlling MAPK signalling cascades during stomatal development in *Arabidopsis*. For this purpose, we first checked the epidermal phenotypes of all five *Arabidopsis MKP* mutants to see if any of these phosphatases has a role in stomatal patterning and differentiation. Based on this finding, I identified and characterized MKP1, one of five MKPs, promoting the stomatal cell fate differentiation. In addition, we investigated the role of MKP1 in each different stage of stomatal development using our cell-type specific *in vivo* experiments.

2. Material and Methods

2.1. Plant materials and growth conditions

mkp1-1, *mkp2* and *mkp1NahG* (Received from Dr. Ulm), *ibr5-3* (SALK_039359C), *dsptp1-1* (SALK_082811C), *phs1-5* (SALK_070121C), *ap2c3* (also known as *pp2c5*, SALK_109986), *ptp1* (SALK_118658), *sid2* (Adie*, et al*., 2007), *yda2* (AT1G63700.1), all these mutant lines are in Colombia ecotype of Arabidopsis.

Seeds were sterilized for 11 min using sterilization solution (30% bleach, 0.19% triton X-100) and sowed on plates of ½ Murashige and Skoog (MS) medium mixed with 1% of sucrose and 0.35% agarose gel (BioShop). Seeds were incubated at 4 °C in dark for 2 to 5 days. Germination proceeded at 21°C using 16h/8h light/dark (long day) cycle in a standard growth chambers (E15 Conviron) for 7 to 10 days before analyses. For phenotypic assays, 10 days seedling were transplanted into soil mixture (2 black earth: 1 vermiculte: 1 peat moss) and grown in the same condition.

Single mutants were crossed in order to generate double mutants. Genomic DNA was prepared following the protocol as described (Edwards *et al*., 1991). Genotyping of the F2 generation was conducted using PCR to obtain double homozygotes. Table 2 to 6 listed the primers used to identify T-DNA insertions and gene specificity.

2.2 Plasmid construction and generation of *Arabidopsis* transgenic plants

Table 1 lists the plasmid constructs generated in this study. *MKP1* coding sequence containing introns was used to generate *MKP1::MKP1* (pJSL120) construct. For expression with the *SPCH, MUTE,* and *FAMA* promoters, each promoter::MKP1 construct was combined into pR4 501 using Gateway LR recombinase II (Invitrogen).

For *MKP1::MKP1* (pJSL120), LR reaction used to combine *MKP1* in pKUT612 (pJSL18) and *MKP1pro* in pENTR5'-Topo TA (pJSL118) into pR4 501.

For *SCRM::MKP1-GFP* (pJSL125), LR reaction used to combine *SCRMpro* in pENTR 5' TOPO (pAR130) and *MKP1* in pKUT612 (pLGC9) into pR4 504.

For *SPCH::MKP1-GFP* (pJSL117), LR reaction used to combine *SPCHpro* in pENTR 5'TOPO (pAR175) and *MKP1* in pKUT612 (pLGC9) into pR4 504.

For *MUTE::MKP1-GFP* (pJSL119), LR reaction used to combine *MUTEpro* in pENTR5'TOPO (pAR202) and *MKP1* in pKUT612 (pLGC9) into pR4 504.

For *FAMA::MKP1-GFP* (pJSL121), LR reaction used to combine *FAMApro* in pENTR5'-Topo TA (pKMP154) and *MKP1* in pKUT612 (pLGC9) in pR4 504.

MPK3 RNAi plasmid was constructed by Dr. Longgang Cui.

Primer design was done with the help of Dr. JinSuk Lee and PCR was ran using T100™ Thermal Cycler (Bio-Rad). E.Z.N.A.® Gel Extraction Kit (Omega) is used to purify target DNA band from agarose gel. Gene JET PCR Purification Kit (Thermo Fisher) is used to purify PCR product. Top 10 *E. coli* strain were used for transformation and proliferation of constructed plasmid and E.Z.N.A.® Plasmid Mini Kit I (Omega) is used for plasmid purification. For LR Reaction (Gateway system), DreamTaq DNA Polymerases kit (Thermo Fisher) was used. *Agrobacterium tumefaciens* (GV3101) were transformed with the plasmid constructs. Floral dip method (Clough and Bent, 1998) was used to transform *mkp1* mutant *Arabidopsis* plants. T1 seeds from dipped plants were harvested, sowed and screened on antibiotics ½ MS plates. Homozygous lines with one insert at T3 were obtained.

2.3 Floral Dip Method

Floral dip was performed as described (Clough and Bent, 1998). Here 5 ml of culture of *Agrobacterium tumefaciens* GV3101 carrying constructs in LB medium and appropriate antibiotics was grown overnight at 28°C at 200 rpm. The culture was then added to 400 ml to make a main culture, which was grown overnight under the same conditions. The cultured *Agrobacterium* were collected by centrifugation at 4000 x g 4 °C for 10 minutes. The pellet was then resuspended in 400 ml of dipping buffer (5% sucrose containing 0.05% Silwet L-77). Flowering *Arabidopsis thaliana* plants with approximately 10 cm bolts were dipped into the *A. tumefaciens* for 5 sec twice. Dipped plants were kept in dark condition for 24 hours and then transferred back to normal growth chamber conditions. The T1 seeds from dipped plants were harvested and sowed for screening using antibiotics in ½ MS plates to get homozygous lines with

one insert at T3.

2.4 Agro Transformation

Plasmid DNA (1.5 − 2 µl) was used to transform GV3101 competent cells. After transformation, agrobacteriawere then kept on ice for 5 min prior to electroporation. Electric chock was introduced using Micro Pulser Electroporator (Bio-Rad). One millilitre of SOC buffer was added and incubated at 28°C for 1hr at 120 rpm. Cells were then spread on LB plates with appropriate antibiotics for selection. After 2 days incubation at 28°C, colonies of transformed agrobacteria were cultured for further floral dipping.

2.5 Toluidine Blue O (TBO) Staining

After surface sterilization and imbibition at 4°C for 2 to 3 days, seeds were plated on ½ MS medium with 0.8% agar. Plates were incubated in a growth chamber at 21°C under 16hrs light/8hrs dark (100 µmol m^{-2} s^{-1}). Cotyledons of 10-day-old seedlings were fixed in 1 ml of 9:1 ethanol: acetic acid overnight or until samples were imaged. Samples were subsequentially transferred to 70%, 50%, 25% ethanol, and then ddH_2O with at least 30 minutes intervals. Staining was performed by emerging the samples in staining buffer (0.5% Toulidine Blue in ddH_2O) for 2 min and 15 sec. TBO stain was removed and samples were immediately washed several times with ddH_2O. Imaging was conducted using Nikon Eclipse TiE inverted epifluorescence microscope (15x and 20x magnification).

2.6 Confocal Microscopy

Epidermis images of cotyledons and mature stems and rosette leaves were taken using a Nikon C2 laser scanning confocal microscope with a 40x bright objective lens. To visualize epidermal cell outlines, samples were mounted in diluted propidium iodide solution (excitation, 555 nm; emission, 570-610 nm). Fluorescence of GFP was simultaneously captured (excitation, 488 nm; emission, 543 nm).

2.7 Mature Plants Imaging

Plants were grown in standard conditions (21°C, 16hrs light/8hrs dark) with three biological replicates for each assay. 14-day-old samples were prepared and only the upper part of the seedlings were used (just after the hypocotyl). For 37 and 40-day-old plants, whole plant grown in soil were taken and roots were directly mounted with water. All images were taken using Galaxy Note 5 cellular phone SM-N920W8.

2.8 Tables

Table 1. List of plasmids constructed in this study and their description

Constructs	**Plasmid Name**	**Description**
pSPCH::MKP1-GFP	pJSL117	cell type-specific rescue experiment
pMUTE::MKP1-GFP	pJSL119	cell type-specific rescue experiment
pMKP1::gMKP1	pJSL120	complementation experiment
pFAMA::MKP1-GFP	pJSL121	cell type-specific rescue experiment
pSCRM::MKP1-GFP	pJSL125	cell type-specific rescue experiment
gMKP1 in pKUT612	pJSL18	phosphatase activity experiment
pMKP1 in pENTR5'-Topo TA	pJSL118	phosphatase activity experiment
MKP1 NS in pKUT612	pLGC9	phosphatase activity experiment
pMKP1::gMKP1^{C235S}	pJSL126	phosphatase activity experiment
Est: MPK3 RNAi	pLGC 23	Estradiol (Est) inducible *MPK3* RNAi

(p): promoter, (g): genomic sequence

Table 2. List of primers and their DNA sequence used for genotype analysis

primer	Sequence (5' to 3')
MKP2 1 XhoIf	CGCCTCGAGATGGAGAAAGTGGTTGAT
MKP2 932.rc	CATCAGGTAAGCCACCACTATAG
mkp2-2 LP	TGTCTTAACCGTTGCTGTGG
mkp2-2 RP	CTGGTTTGGGTATGGGATTG
mkp2-3 LP	TGAAACCATCTAATTCCCAGC
mkp2-3 RP	GGTTTACTTCATCTCGGGAGC
mkp1-1f	ACAAGTCTATGGAAGAAGC
mkp1-1.rc	TGTCTTTCGCCACAGCATC
ptp1-1 LP	AGAAAAGGTCAGATTCTCCGC
ptp1-1 RP	TAGTCCCCGCATTTAACAGTC
ibr5-3 f	TCTCCTTCTTCTCCGTCCTTC
ibr5-3 rc	TCTCCGTTGTTTAACCCACTG
ap2c3 f	ACGTATGCTTGGTTACCATGC
ap2c3 rc	CAAGCAAATGAGCTCAAAAGG
phs1-5 f	CTCCACCTTTACCGGATCTTC
phs1-5 rc	AGAGAGCTGCTGCAGCTCTAG
dsptp1-1 rc	AAACAATGACAGCCCATGAAC
dsptp1-2 f	ACCAGATGGGAGAAGAATACAGAG
mpk3-1 f	CTTCTGTTGAACGCGAATTGCG
mpk3-1 .rc	TCCGTTGATGCAAGTTGAGCC
mpk6-2 .rc	CACTGTCGGGAACTTATCAGTGA
mpk6-2 f	GATCTTTTCCATCTGCGTCAAG

primer	Sequence (5' to 3')
pGKB5_GusRb1	ACGCAGCACGATACGCTGG
LBa1	TGGTTCACGTAGTGGGCCATCG
LBb1.3	ATTTTGCCGATTTCGGAAC
SAIL_LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
SAIL_LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
NahGf	GAAAAACAATAAACTTGGCTTGCG
NahG.rc	ACCTTCCAGCACATGACTACG
SID2 2516f	AGACGACCTCGAGTTCTCTATC
SID2 3135 rc	TCGACTAGAGCTGATCTGATC
SPCH 1f (GW)	CACCATGCAGGAGATAATACCG
SPCH 1281.rc	AACCTGAAGAATCTCAAGAGCC

3. Results

3.1. Identification of phosphatase controlling stomatal development

To identify phosphatase(s) that play a role in controlling stomatal development, we examined the distribution and the number of stomata on cotyledons of five Arabidopsis MKP mutants as well as PTP1 and AP2C3 previously known to control MPK3 and/or MPK6 activity (Bartels *et al*., 2009; Umbrasaite *et al.,* 2010).

None of these single phosphatase mutants showed any defect in the epidermis cells development except *mkp1* mutant plant (Fig. 3A to 3H). The epidermis of *mkp1* seedlings occasionally developed clusters of small cells that failed to differentiate into stomata which thus resulted in a lower number of stomata. (Brackets in Fig. 3B).

Then stomatal index was quantified as the percentage of the number of stomata to the total number of cells for all these mutants (stomatal index). As shown in figure 3I, we observed that the *mkp1* mutant display statistically significant reduction in stomatal index compared with wild type and any other single phosphatase mutants at the early stage of development (10 dpg).

As mentioned before, *mkp1* growth phenotypes appeared after 2 weeks after germination (Bratel S, *et al.,* 2009). To examine if these defects are relevant to the stomatal phenotype in the *mkp1* mutant, we checked the morphology of 14 dpg seedlings for all mutants just before the appearance of defected growth phenotypes caused by *mkp1* mutant. Figure 3J illustrated that the growth phenotypes of any of the mutants were indistinguishable from the wild type. This result suggests that the morphological defects in mature *mkp1* mutant plant was not the cause of *mkp1* epidermis phenotype since *mkp1* mutant shows arrested epidermis cells even before the appearance of the growth alteration.

These results confirmed that *mkp1* mutation reduced the number of stomata by altering the formation of mature stomata.

Figure 3.

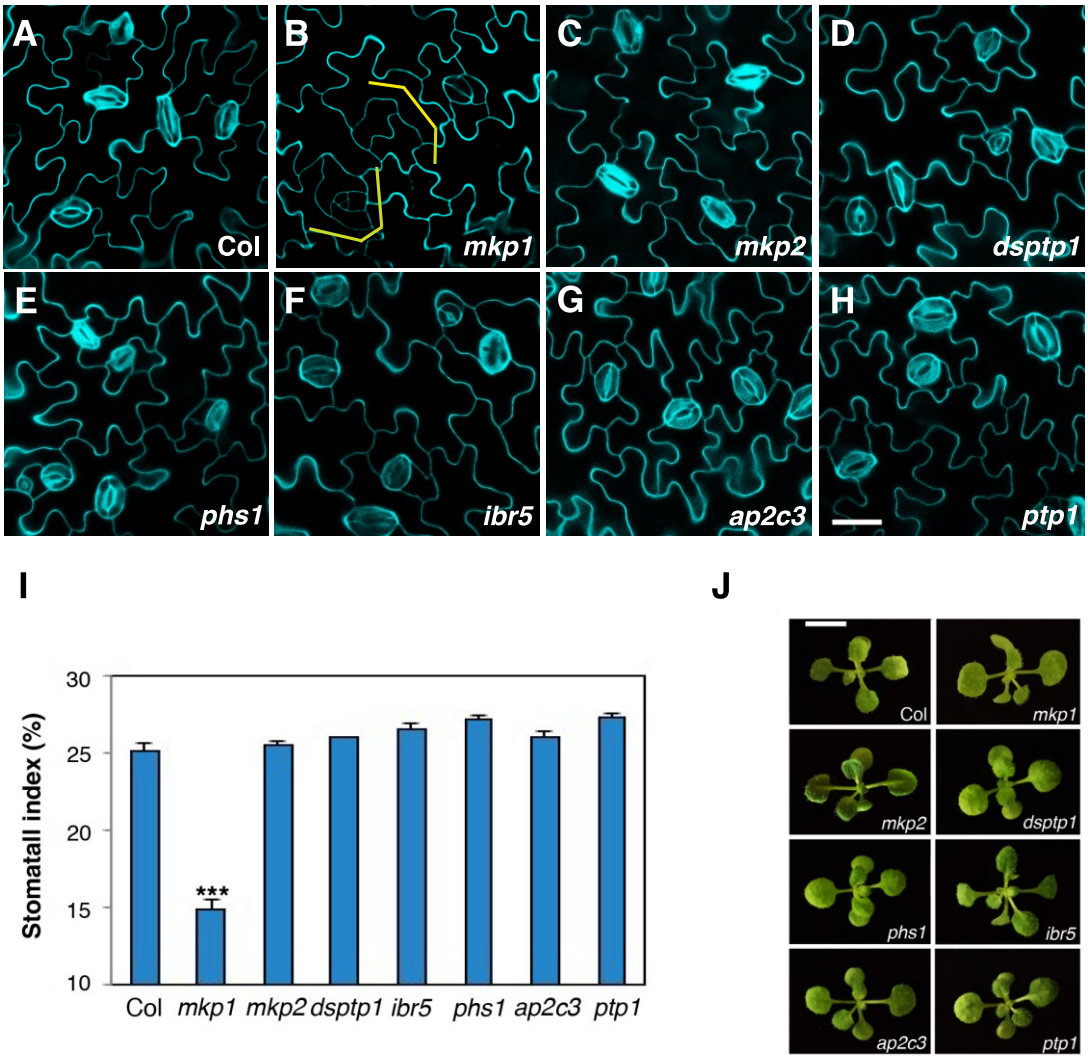

A
Col
B
mkp1
C
mkp2
D
dsptp1
E
phs1
F
ibr5
G
ap2c3
H
ptp1
I
Stomatall index (%)
30
25
20
15
10
***
Col
mkp1
mkp2
dsptp1
ibr5
phs1
ap2c3
ptp1
J
Col
mkp1
mkp2
dsptp1
phs1
ibr5
ap2c3
ptp1

Figure 3. Stomatal development defects in *mkp1* mutants.

(A-H) Representative confocal images of 10-days-old abaxial cotyledons of the following genotypes: wild type Col (A), *mkp1* (B), *mkp2* (C), *dsptp1* (D), *phs1* (E), *ibr5* (F), *ap2c3* (G) and *ptp1* (H). The *mkp1* single mutant, but not other phosphatase mutants, shows stomatal development defects (less stomata and islands of small arrested cells (brackets; B). Cells were outlined by propidium iodide staining and images were taken under the same magnification. Scale bar = 30 μm.

(I) Quantitative analysis of 10-days-old abaxial cotyledon epidermis. Stomatal index (SI) is expressed as the percentage of the number of stomata to the total number of epidermal cells. Asterisks above the column indicate significant difference compared with data from wild type plants. (*** $p<0.0001$, Student's t-test). n = 14-15 for each genotype. The experiments were repeated three times with similar results. Bars, means. Error bars, s.e.m.

(J) The phenotypes of 2-week-old seedlings of indicated genotypes grown on ½ MS plates. Scale bar in Col = 0.5 cm and others are at the same scale. All phosphatase mutants exhibit similar growth morphology as the Col. The representative images were selected from at least five replicates.

3.2 *mkp1* mutant phenotype prevails above ground organs.

Stomata are known to be present on all above ground organs of the plants except for petals and stamen. To investigate if the *mkp1* epidermis phenotype is shown on other organs, we examined *mkp1* mutants at 7 weeks post germination.

Interestingly, an obvious reduction in stomata was detected with arrested cells in *mkp1* mutant stems as compared to the wild-type (Figure 4D to 4F). This reduction was significant in certain T-DNA insertion lines, which showed only pavement cells and arrested stomatal cells without mature stomata (Figure 4E).

Besides stems, rosette leaves of the mature *mkp1* plant were also examined. As expected, the reduction of stomata was significant (Figure 4A to 4C). It is noteworthy that in the rosette leaves the arrested cells became larger in size and lobular in shape, which resembled a small pavement cells (Figure 4A to 4C).

Taken together, these data confirmed that the epidermis phenotype caused by *mkp1* mutation prevails the upper ground organs of the plant.

Figure 4.

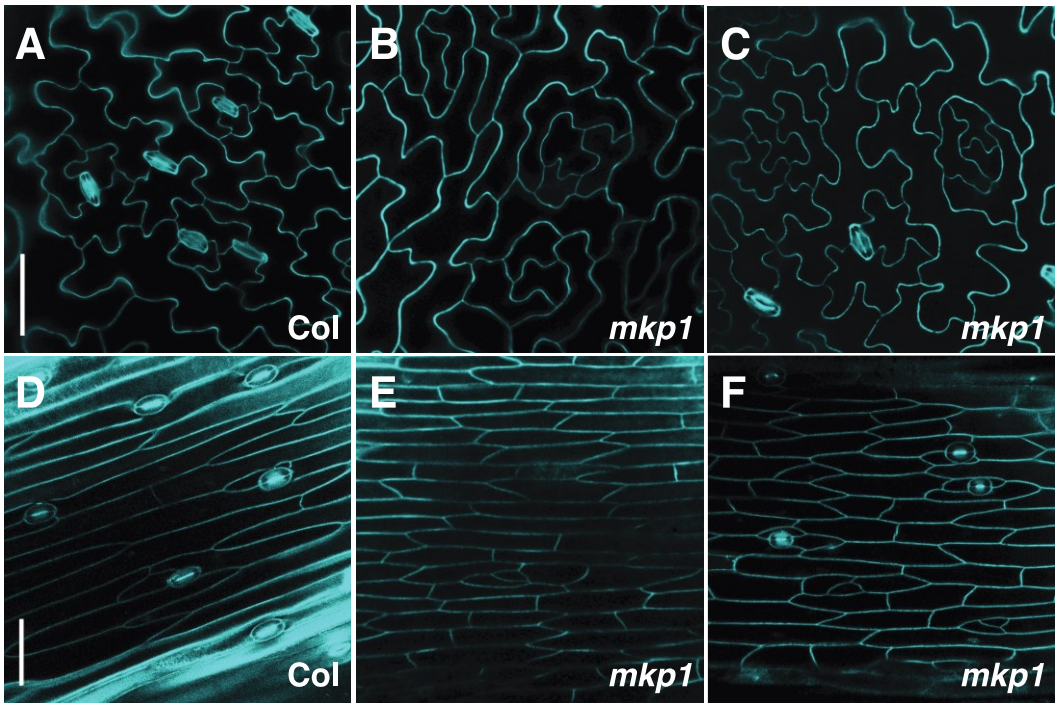


Figure 4. *mkp1* mutant phenotype prevails upper ground organs.

(A) to (C) 33 – 36 dpg rosette leaf epidermis images under the same magnification (40x). (A) Col, (B) *mkp1* 33dpg, (C) *mkp1* 36dpg. The *mkp1* single mutant, but not Col wild type, still shows stomatal development defects even in the late stage. Less stomata and islands of arrested cells are obvious. Scale bar = 30 μm

(D) to (F) 33 – 36 dpg stem epidermis images using same magnification (40x) (A) Col, (B) *mkp1* 33dpg, (C) *mkp1* 36dpg. The *mkp1* single mutant, but not Col wild type, still shows stomatal development defects (less stomata and islands of arrested cells) scale bar =30 μm

3.3 Specific role of MKP1 in controlling stomatal development

We further generated all combinations of double knockout mutant plants to uncover possible redundant roles of MKP1 with other MKPs as well as PTP1 and AP2C3 during stomatal development.

Direct analysis for these double mutants revealed undistinguishable epidermis between these mutants compared to *mkp1* single mutant. In another word, neither additive nor distinct phenotype is obtained but clusters of small cells failed to differentiate into stomata as in *mkp1* single mutant. (Figure 5A). This data suggested that no redundancy of function between these phosphatases and MKP1 at the level of double mutant during stomatal development.

To confirm this result we generated a statistical data of stomatal index of all these double mutant and we compared it with *mkp1* single mutant and Col. Data revealed no significant differences between these double mutant and *mkp1* single mutant (Figure 5B). In addition to 10-day-old seedings, 14-day-old seedlings were examined. As shown in Figure 5J, no significant morphological alternations were detected between double phosphatase mutants and *mkp1* single mutants.

This significant decrease in stomata number in all double mutants to a level similar that of the *mkp1* single mutant indicates either a major or specific role of MKP1 in controlling stomatal development (Fig. 5I). All together these results do not eliminate the probability of redundancy between these phosphatases during stomatal development, which may appears in triple (in progress) or even quadrat mutants.

Figure 5.

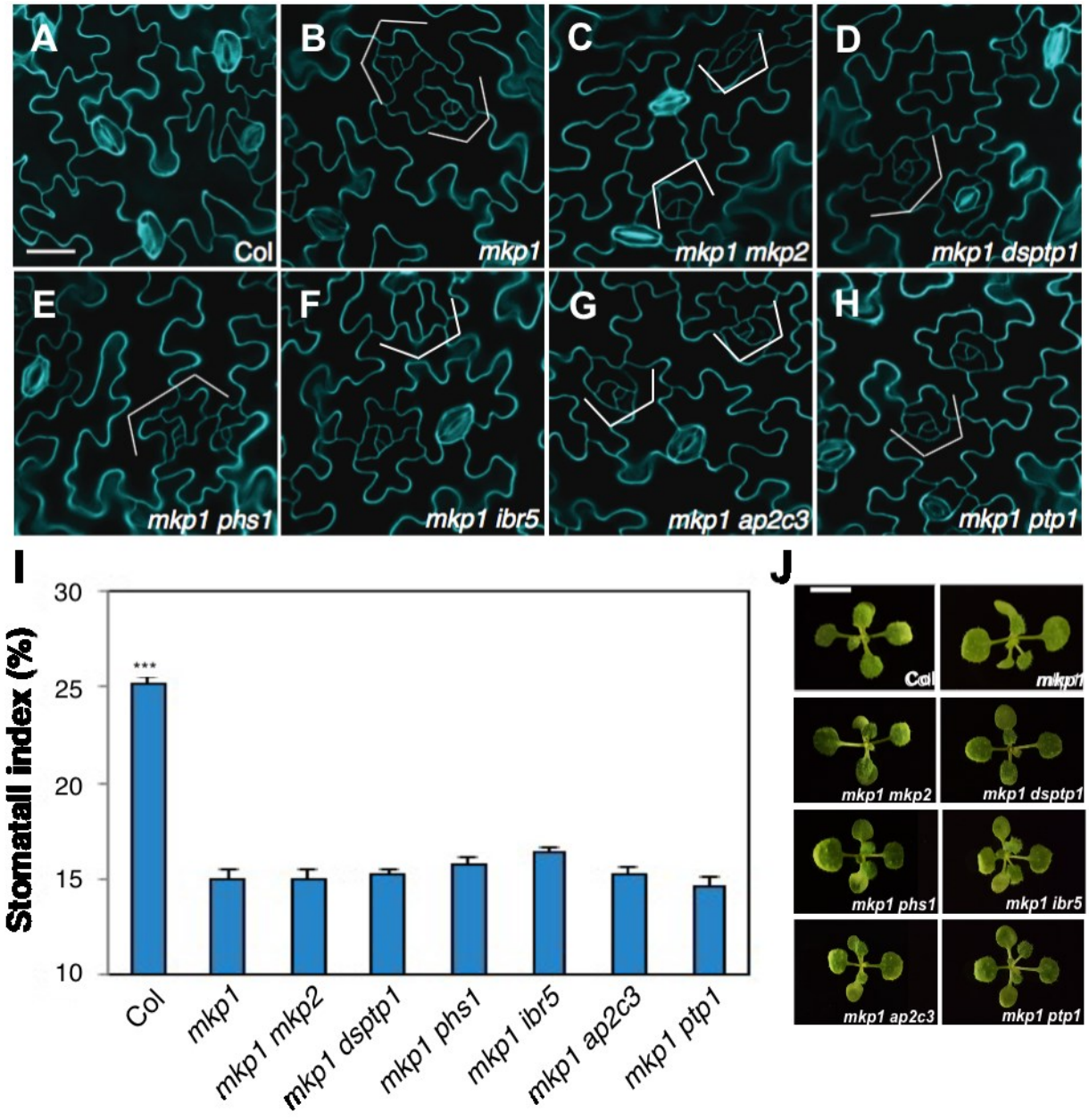

A
Col
B
mkp1
C
mkp1 mkp2
D
mkp1 dsptp1
E
mkp1 phs1
F
mkp1 ibr5
G
mkp1 ap2c3
H
mkp1 ptp1
I
Stomatall index (%)
30
25
20
15
10
***
Col
mkp1
mkp1 mkp2
mkp1 dsptp1
mkp1 phs1
mkp1 ibr5
mkp1 ap2c3
mkp1 ptp1
J
Col
mkp1
mkp1 mkp2
mkp1 dsptp1
mkp1 phs1
mkp1 ibr5
mkp1 ap2c3
mkp1 ptp1

Figure 5. Specific role of MKP1 in controlling stomatal development

(A-H) Representative confocal images of 10-days-old abaxial cotyledons of the following genotypes: wild type *Col* (A), *mkp1* (B), *mkp2mkp1* (C), *dsptp1mkp1* (D), *phs1mkp1* (E), *ibr5mkp1* (F), *ap2c3mkp1* (G) and *ptp1mkp1* (H). The *mkp1* single mutant and all double mutants, but not Col wild type, shows stomatal development defects. Images were taken under the same magnification. Scale bar =30 μm.

(I) Stomatal index% of 10-days-old Abaxial cotyledon epidermis of double mutants. At least 15 samples for each mutant were included in TBO staining. Stomatal index was calculated according to the following equation (SI = NS/NE x 100) where SI is the stomatal index%, NS is number of stomata and NE is the number of total epidermis cells. Stars indicate the significant variation in stomatal index % in Col wild type compared to *mkp1* mutants.

(J) 14-days-old seedlings images of the double phosphatase mutants show no distinguishable phenotype compared to Col and *mkp1*. Seedlings were grown under standard conditions.

Scale bar =50 μm.

3.4 Complementation of *mkp1* loss-of-function mutants by *MKP1*

To determine whether the inhibited stomatal development in *mkp1* mutant was caused by the loss of MKP1, we generated transgenic *Arabidopsis* plants expressing *MKP1* in the *mkp1* mutant background under its own native promoter for complementation analysis.

Three homozygous lines were tested. As shown in Figure 6A , stomatal development in these transgenic lines was fully restored to that observed in wild type, suggesting that fewer stomata and more non-stomatal cells found in *mkp1* plants were complemented by the *MKP1* transgene.

Furthermore, the stomatal index was quantified for these lines. As shown in Figure 6B, stomatal index is rescued in these lines to the same extent comparable to wild type, confirming that the reduction of stomatal number in *mkp1* mutant is reversed with after the expression of native protein of MKP1.

This complementation was not only observed at the level of stomata, but also expanded to recover the morphological alteration caused by *mkp1* mutant. Figure 6C indicates that although there was a mild variation in plant height between three complementation lines, the rescued phenotype was significantly different from that of *mkp1* mutants.

All together these results suggested that the defect in cell development on the plant epidermis is caused by the null single mutation of *MKP1* gene.

Figure 6.

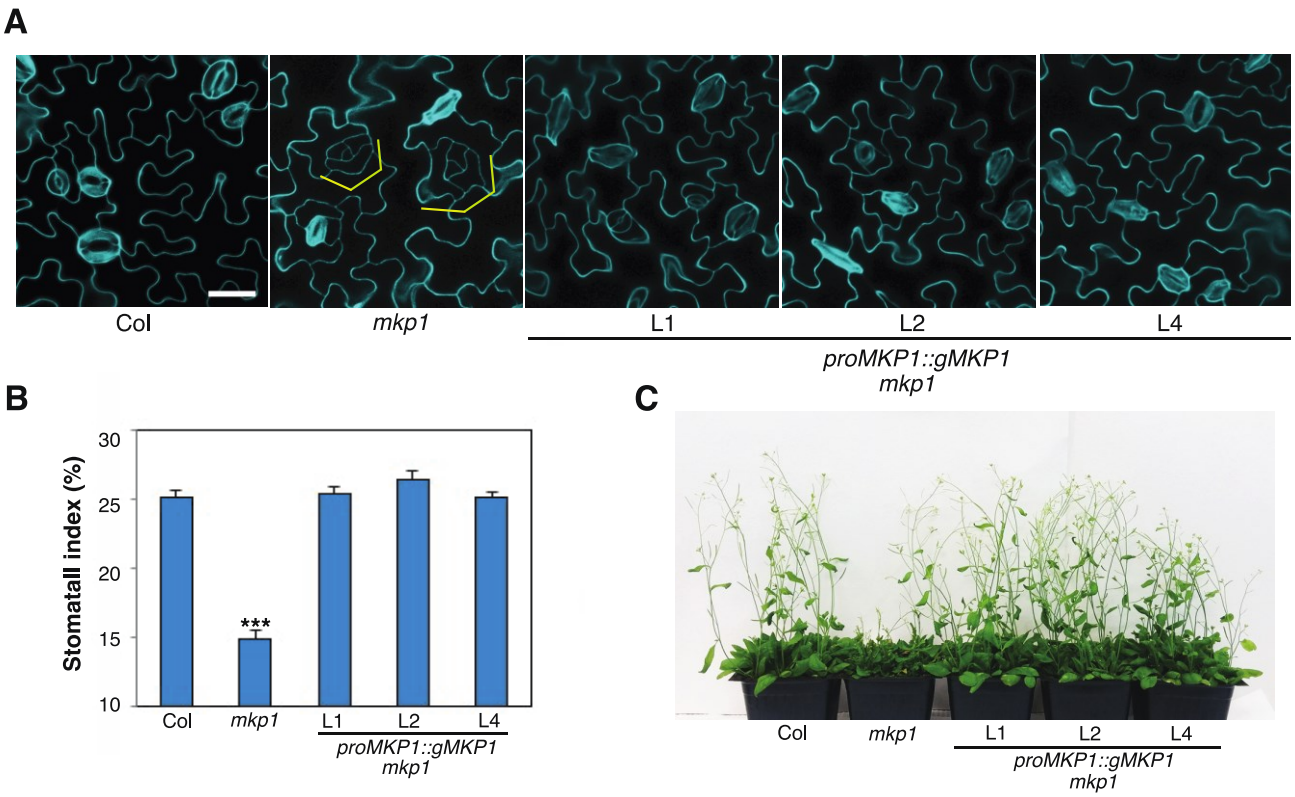

A
Col
mkp1
L1
L2
L4
proMKP1::gMKP1
mkp1
B
Stomatall index (%)
30
25
20
15
10
***
Col
mkp1
L1
L2
L4
proMKP1::gMKP1
mkp1
C
Col
mkp1
L1
L2
L4
proMKP1::gMKP1
mkp1

Figure 6. Complementation of *mkp1* loss-of-function mutant by MKP1.

(A) 10-day-old Abaxial cotyledon epidermis of *Col*, *mkp1*, and Line 1, 2, 4 of *proMKP1::g MKP1* in *mkp1* respectively (T3 homozygous lines with one insert). Stomatal developmental defect is fully restored in transgenic lines to a level comparable to the wild type. scale bar =30 μm.

(I) Stomatal index% of 10-day-old Abaxial cotyledon epidermis, at least 15 samples for each line were included in TBO staining. Stomatal index was calculated according to the following equation (SI = NS/NE x 100) where SI is the stomatal index%, NS is number of stomata and NE is the number of total epidermis cells. Stars indicate the significant decrease in stomatal index % *in mkp1* mutant compared to the wild type and the complementation lines1,2 and 4.

(J) 40 dpg mature plants image of the three complementation lines compared to *Col* and *mkp1* grown at standard conditions. Growth recovery occurred in *mkp1* due to the expression of MKP1 transgene in three complementation lines.

3.5 A mutation in a conserved residue in the catalytic active site of MKP1 abolishes its function in stomatal development

All dual specificity phosphatases have an essential amino acid located in the catalytic center. Modification at this location by replacing cysteine with serine is known to abolish the phosphatase activity of MKPs. To determine whether the phosphatase activity of MKP1 is required for its function in stomatal development, we mutated a conserved cysteine residue (C235) in the catalytic active site (V*X*VH**C***X*2G*X*SRS*X*5AYLM) of canonical DSPs to serine.

Several transgenic lines were generated to express *MKP1*^{C235S} in the *mkp1* background under its native promoter (Figure 7). Epidermal Analysis of these transgenic lines showed clusters of small cells that failed to differentiate into stomata, a phenotype resemble that of the *mkp1* single mutant (Figure 7).

These results suggested that *MKP1*^{C235S} transgene was unable to complement the phenotype of *mkp1* mutants. This indicates that the phosphatase activity of MKP1 is essential and crucial to provide the proper function during stomatal development.

Figure 7.

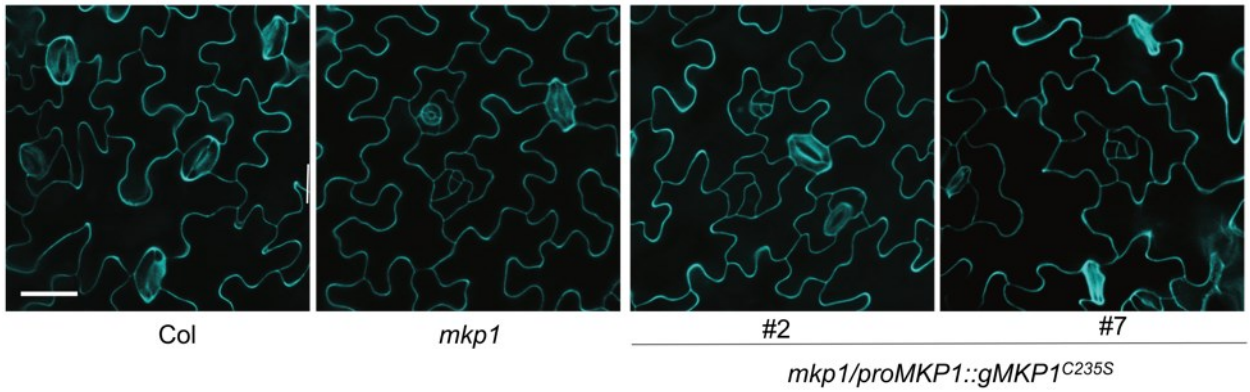


Figure 7. The function of MKP1 during stomatal development requires the catalytic active site cysteine

10-day-old Abaxial cotyledon epidermis of *Col*, *mkp1*, and Line 2 and 7 of *proMKP1::* $MKP1^{C235S}$ in *mkp1* respectively (T3 homozygous lines with one insert). Stomatal developmental defect is not rescued in *mkp1* expressing *proMKP1::* $MKP1^{C235S}$. Scale bar =30 μm.

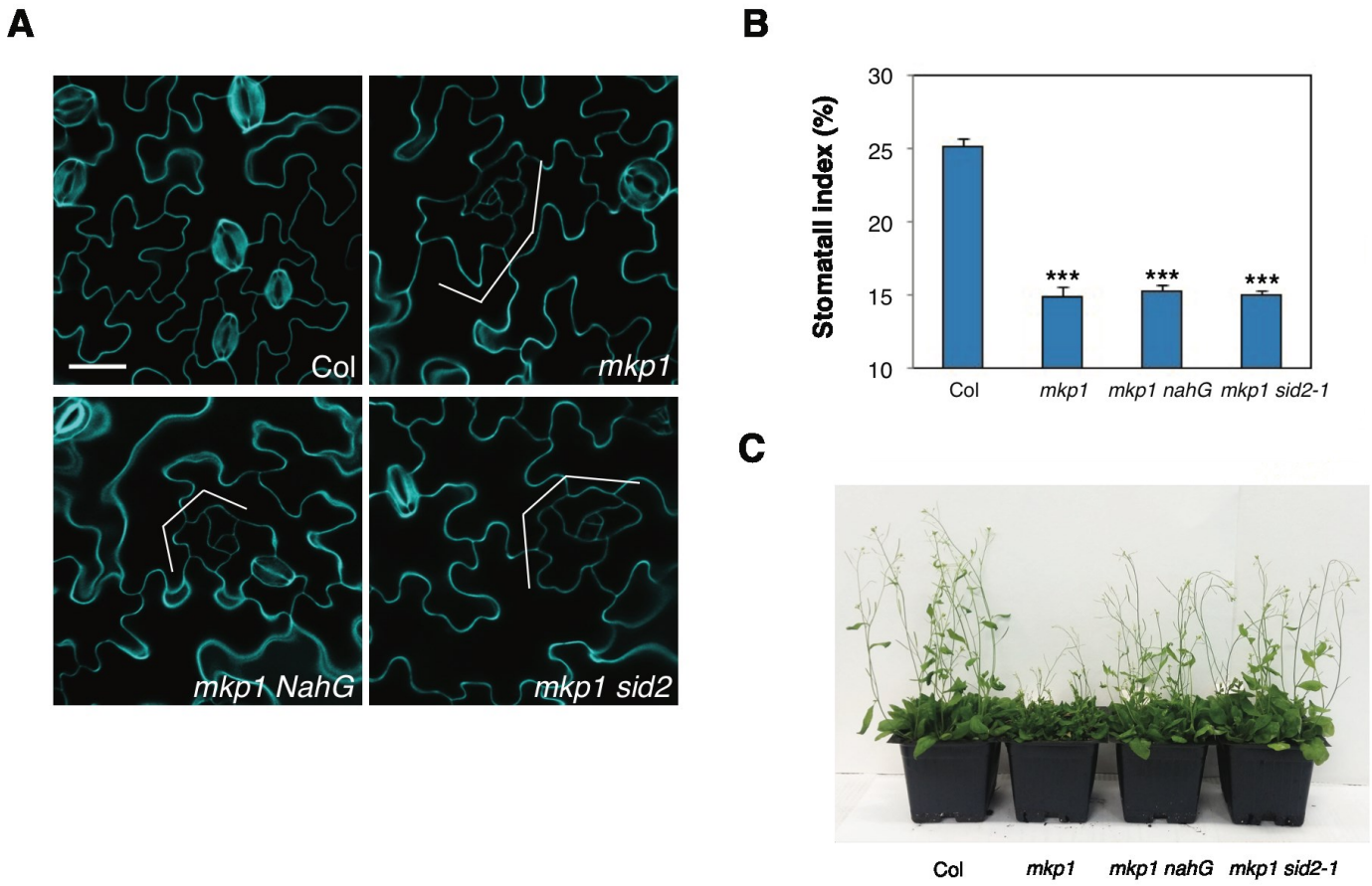
3.6 *mkp1* Epidermal Phenotype is not the result of Salicylic Acid Accumulation

Previous studies showed that *mkp1* mutants induced hyper accumulation of SA (Bartels et al., 2009). In order to investigate whether *mkp1* stomata phenotype was induced by elevated levels of SA, *NahG* integration (SA–degrading hydroxylase) (Suzuki *et al*., 2000) and *sid2* mutation (SA biosynthesis) were used in the genetic studies.

Expression of *NahG* and *sid2* mutation dramatically complemented tthe *mkp1*'s defense-related growth defects (Fig. 8C). However, a substantial decrease in stomata number with arrested small cell clusters was still observed in both *mkp1NahG* and *mkp1 sid2-1* double mutants, which resembled the phenotype in *mkp1* single mutant (Fig.8A).

Furthermore, the stomatal index was quantified these plants. As shown in Figure 8B, stomatal index is not rescued in *mkp1NahG* and *mkp1sid2* and still exhibited a comparable level to *mkp1* mutant. These results clarify that *mkp1* mutant, rather than the consequent SA accumulation, have induced the arrested cells during stomatal development.

Figure 8.


A
Col
mkp1
mkp1 NahG
mkp1 sid2
B
Stomatall index (%)
30
25
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15
10
***
***
***
Col
mkp1
mkp1 nahG
mkp1 sid2-1
C
Col
mkp1
mkp1 nahG
mkp1 sid2-1

Figure 8. *mkp1* stomatal phenotype is not the result of salicylic acid accumulation

(A) 10-day-old Abaxial cotyledon epidermis of Col, *mkp1*, *mkp1sid*, *mkp1NahG*. Stomatal development defects (less stomata and islands of small arrested cells) is obvious in *mkp1* single mutant and the two double mutants. Scale bar=30 μm. Using the same magnification (40x)

(B) Stomatal index% of 10-day-old Abaxial cotyledon epidermis, at least 15 samples for each line were included in TBO staining. Stomatal index was calculated according to the following equation (SI = NS/NE x 100) where SI is the stomatal index%, NS is number of stomata and NE is the number of total epidermis cells. Stars indicate the significant decrease in stomatal index % *in mkp1sid*, *mkp1NahG* and *mkp1* mutant compared to the wild type.

(C) The phenotypes of 36 days-old plants of indicated genotypes. Complementation of the defence-related growth defects in *mpk1NahG* and *mkp1 sid2.*

3.7 Lineage Cell Identity in *mkp1* Mutant Phenotype

After *mkp1* epidermis phenotype was identified and confirmed using complementation assay, we tried to determine the identity of those arrested cells in *mkp1* mutants. For this purpose, new transgenic plants were constructed expressing *TMM::GUS-GFP* in both *mkp1* mutant and *Col* background. As shown previously (Kanaoka *et al*., 2008), *TMM* promoter is active in all stomatal lineage cells but not mature stomata, thus it was used as a lineage-cell specific marker.

Day 3 after germination, GFP signal was detected in all stomatal lineage cells except for mature stomata in the *Col* background (Figure 9). Same expression pattern was detected in the *mkp1* mutant background with an extra location within the arrested cells of the *mkp1* mutant phenotype. At day 11 the signal is disappeared totally in Col which indicate full maturation of stomatal lineage cells, however in *mkp1* mutant the green signal is clearly present indicating that *TMM* promoter is still active in these cells and suggesting that they belong to the stomatal-lineage cells.

These results confirmed the identity of the arrested cells in mkp1 mutants as a stomatal lineage cells and identify the regulation function of MKP1 during the stomatal cell fate differentiation.

Figure 9.

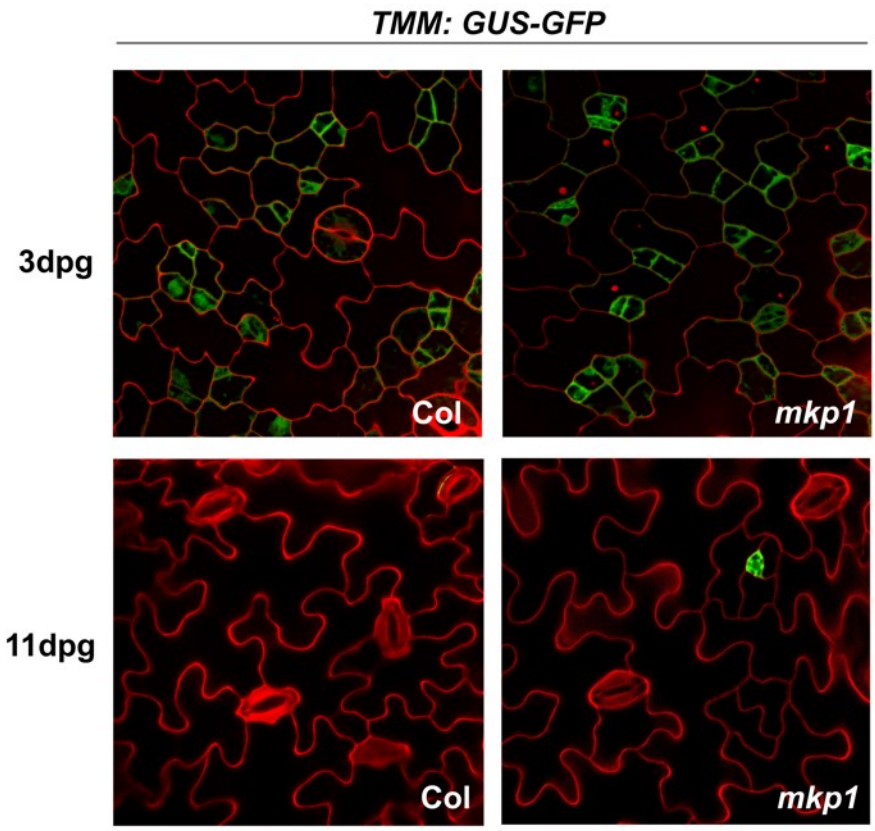

Figure 9. Time-course analysis of stomatal development in the *mkp1* mutant.

Abaxial epidermis images of 3 and 11 dpg wild-type (Col) and *mkp1* cotyledons. *TMM:GUS-GFP* expression (GFP signal) was used to monitor stomatal lineage cells. Some auto fluorescence appeared (red dots). All images were taken at the same magnification (Z1.5 40x). GFP expression was shown in all stomatal lineage cells as well as in the arrested cells of *mkp1* mutants.

3.8 *MKP1* genetically acts downstream of *YDA* to regulate stomatal development

YDA is considered the first player in MAPK signalling cascade, immediately after the ligand-receptor interaction. As mentioned before, *yda* single mutant exhibited severe dwarfism and sterility, which drove us to use heterozygous version of *yda* mutant and cross it with *mkp1*. Interestingly, we were able to generate a double homozygous *mkp1yda* with no defective phenotype. It is known that *yda* epidermis shows clusters of stomata due to its negative role in stomata development. However, this phenotype disappeared in the double mutant, suggesting that *MPK1* gene acts epistatically to *YDA* (Figure 10A and 10B).

Developmental alterations in *yda* mutant start two weeks after germination. We then examined the morphology of *mkp1yda* double mutant at 17 dpg, as compared to *yda* single mutant. Double mutant showed normal growth compared to *yda* single mutant (Figure 10D). This normal growth continued through the life cycle of the plant, suggesting that a complete rescue of *yda* developmental defects in this double mutant (Figure 10E to 10G).

Figure 10.

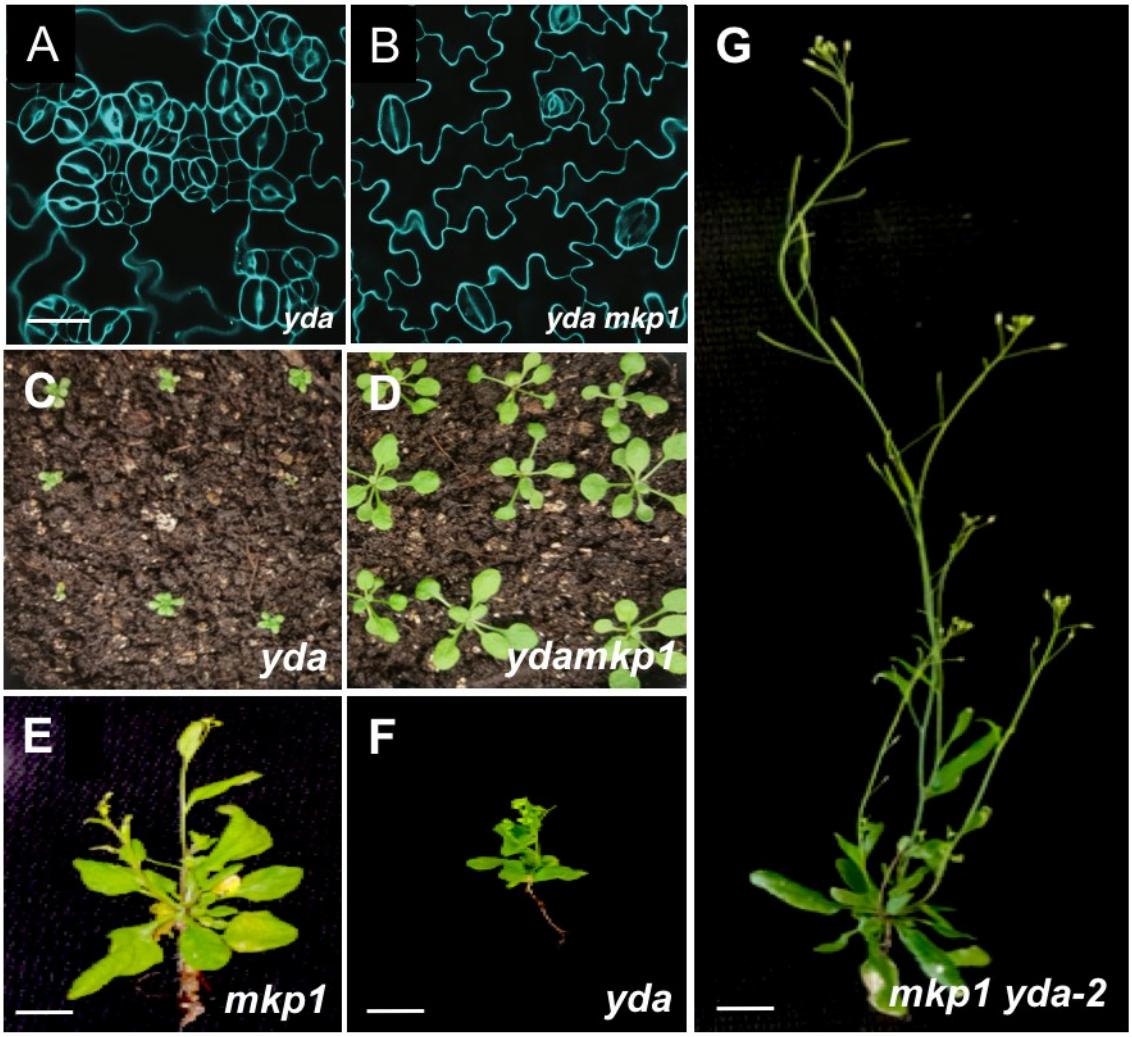

Figure 10. *MKP1* functions downstream of *YDA*

(A) and (B) 7-days-old Abaxial cotyledon epidermis of *yda-2*(A), *mkp1yda-2*(B). Obvious rescue of *yda* phenotype after crossing with *mkp1* scale bar= 25µm

(D) Top view of 17-days-old for *yda-2* (left) *and mkp1yda-2*. Seedlings of *yda* mutant are able to survive after crossing with *mkp1* (right)

(E) to (G) 40 dpg mature plants images of *mkp1*(E), *yda-2*(F), *mkp1yda-2*(G). Obvious recovery in *mkp1yda* plant compared to *yda* and *mkp1* single mutant plants. Scale bar= 1.5cm.

3.9 *MKP1* genetically acts upstream of *MPK3* and *MPK6* to regulate stomatal development

The involvement of MKP1 in stomata development has led us to further examine the genetic interactions between MPK1 and key players of MAPK pathway. *MPK3* and *MPK6* are known to play a crucial role during stomatal development by regulating SPCH so inhibit stomatal production. Single mutants of *mpk3* or *mpk6* has no obvious developmental phenotypes, however, the double mutant shows an embryonic lethality. Previously it was proven that MKP1 interacts with MPK3 and MPK6 but their epistatic location during stomatal development is not clear yet.

To skip the lethality caused by *mpk3mpk6* double mutant and to reveal a clear epistatic location of MKP1 versus MPK3/6, we generated new transgenic plants expressing *MPK3RNAi* under the control of estradiol inducible promoter in *mpk6* single mutant and *mpk6 mkp1* double mutant back ground. In the presence of estradiol, *mpk6* plant behaved as a non-null double mutant *mpk3mpk6* with a cluster of stomata as shown in figure 11A and 11B. However, *mkp1mpk6* double mutant plant converted into non-null triple mutant *mkp1mpk6mpk3* with a copying of double mutant *mpk3mpk6* phenotype.

These results convincingly demonstrated that *MPK3* and *MPK6* are epistatic to *MKP1,* consistent with the predicted function of *MKP1* as a MAPK phosphatase.

Figure 11.

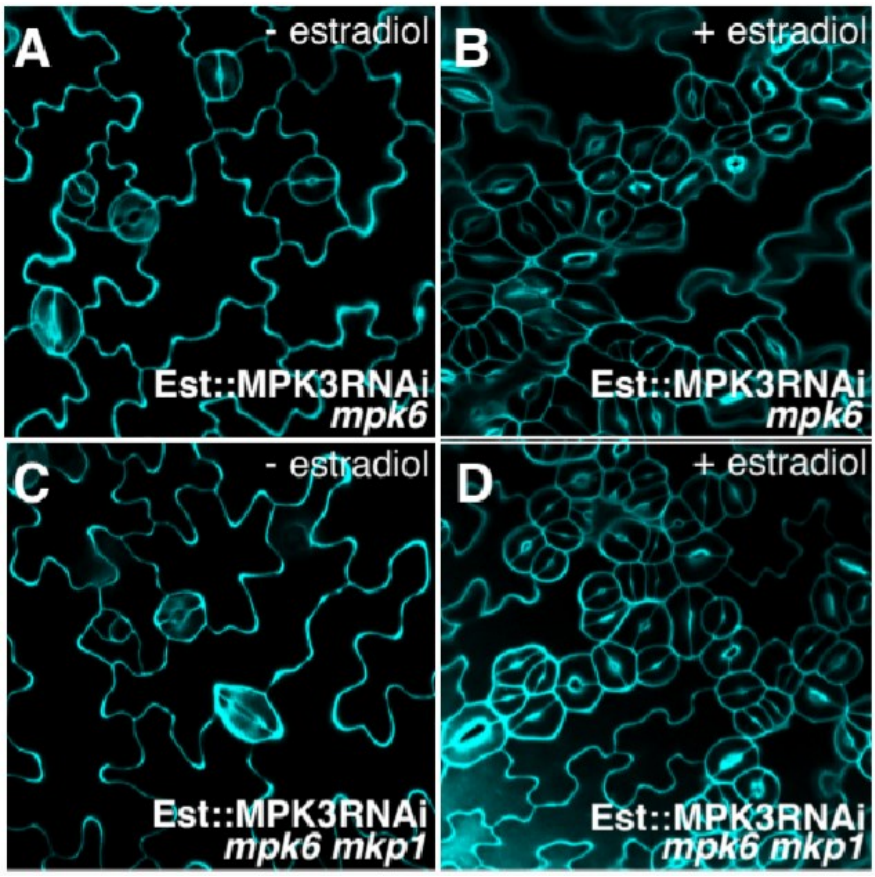
A
- estradiol
Est::MPK3RNAi
mpk6
B
+ estradiol
Est::MPK3RNAi
mpk6
C
- estradiol
Est::MPK3RNAi
mpk6 mkp1
D
+ estradiol
Est::MPK3RNAi
mpk6 mkp1

Figure 11. *MKP1* genetically acts upstream of *MPK3* and *MPK6* to regulate stomatal development

Confocal images of the epidermis of 7 dpg cotyledon. (A, B) *Est::MPK3RNAi* in *mpk6* mutant, –Est and +Est respectively. (C, D) *Est::MPK3RNAi* in *mpk6 mkp1* double mutants, –Est and +Est respectively. MKP1 acts upstream of MPK3 and MPK6. Images were taken at the same magnification (Z2 40x).

3.10 *mkp1* phenotype rescue using stomatal cell-type specific promoters

After the characterization of *MPK* gene in controlling stomatal development and the relevance of its epistatic location versus key transcription factors, important question arises as in which specific stage that *MKP* acts during stomatal development?

For this purpose, complementation assays were performed using stage-specific promoters to drive *MKP1-GFP* expression in the *mkp1* mutant background. *SCRM* is known to be expressed at all stage of stomatal development, which diminishes right before maturation. Results demonstrated that *SCRM:MKP1-GFP* is able to complement *mkp1* stomatal phenotype with normal stomatal spread and no arrested cells on the epidermis (Figure 11A to 11C). Consistent with the *SCRM* expression, MPK1-GFP appeared at all stomatal lineage cells except for mature stomata (Figure 11E). This indicates that expression of *MKP1* gene at the stages where *SCRM* is expressed was able to complement the stomatal defect caused by *mkp1* single mutant. This result was considered as a strong and additional confirmation of MKP1 involvement in stomatal development.

Next, complementation assays was performed using the *SPCH* promoter, which is expressed specifically during stomatal initiation stage. As shown in Figure 11A, 11B and 11D, *SPCH:MKP1-GFP* successfully restored the abnormal stomatal phenotype of *mkp1* mutant. As expected, GFP signal was detected in MMC and meristemoids. These results demonstrated that *mkp1* stomatal defect was complemented using a *MKP1* coding sequence containing introns under the control of *SPCH* promoter, suggesting that the defects in stomatal development within *mkp1* single mutant occurred at the initial stage where SPCH is acting. Primary results from targeting second and third stage of development revealed no complementation of *mkp1* stomatal phenotype (Data not shown, in progress).

Figure 12.

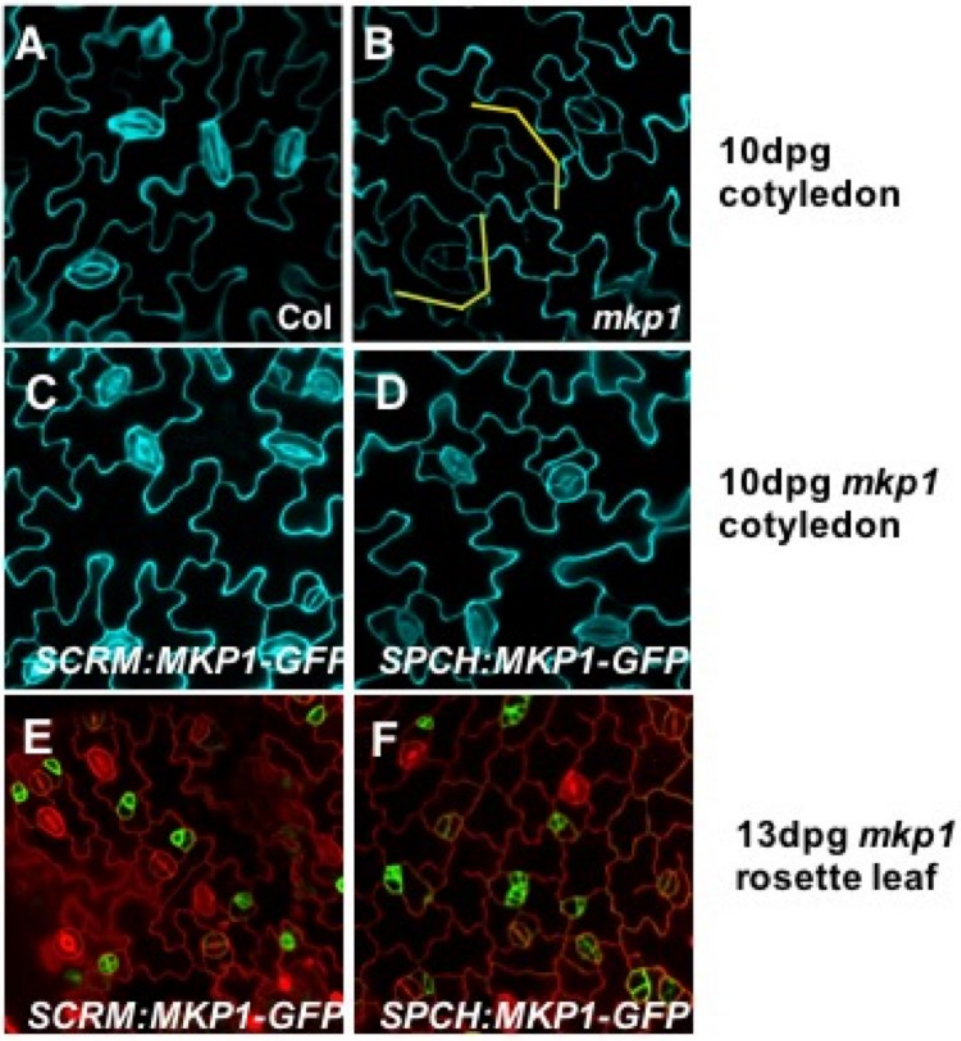
A
Col
B
mkp1
10dpg
cotyledon
C
SCRM:MKP1-GFP
D
SPCH:MKP1-GFP
10dpg mkp1
cotyledon
E
SCRM:MKP1-GFP
F
SPCH:MKP1-GFP
13dpg mkp1
rosette leaf

Figure 12. *mkp1* complementary assay using Transcription Factor promoters

(A) to (D) 10 dpg cotyledon abaxial epidermis images of (A)Col, (B)*mkp1,* (C)*mkp1* expressing *SCRM:MKP1-GFP,* (D)*mkp1* expressing *SPCH:MKP1-GFP*.

(E) 13 dpg abaxial epidermis of first rosette leaf of *mkp1* expressing *SCRM:MKP1-GFP*

(F) 13 dpg abaxial epidermis of first rosette leaf of *mkp1* expressing *SPCH:MKP1-GFP*

Images were taken at the same magnification (Z2 40x). Scale bar =25μm

4. Discussion

Stomata development on the epidermis of the plants starts from precursor cells called protoderm. Differentiation of these cells into mature stomata crosses through several stages, which relies on the function of three transcription factors SPCH, MUTE and FAMA with the coordination of SCRM (Peterson *et al.,* 2010). Stomatal density and spacing are controlled by developmental and environmental cues through intracellular signaling pathways (Serna, 2009). This intracellular signaling started from sequential MAPKs activation upon cell surface ligand-receptor interaction. It is clear that this signaling cascade ends with a negative effect on the initiation step of stomatal differentiation. Therefore, continuing signaling transduction in this pathway resulted in an excess of cells entering into stomatal-lineage cells. To avoid this alteration, plants modulate a system to balance between activation through phosphorylation and deactivation through dephosphorylation of the MAPKs. It is known that the phosphatase is the main protein that deactivates MAPKs through dephosphorylation. For this reason, we searched among the dual specificity phosphatases and identified MKP1 as a positive regulator of stomatal development through its effect on SCRM and SPCH via MPK3/6.

4.1 *mkp1* knockout mutation arrests and decreases stomatal number on all plant epidermal surfaces during the whole plant life.

Negative regulation by MAPK signaling on the initiation of stomatal differentiation indicates that the increase in the activity of the MAPKs will arrest protodermal cell differentiation toward stomata, and thus directing cell fates toward pavement cells. Our results confirmed this finding, in which the loss-of-function mutation of *MKP1* was able to liberate and enhance the negative activity of MPK3/6, and therefore arrest the cell cycle and proliferation. We were able to characterize *mkp1* mutant through the complementation assay. The results of this assay were confirmed using several complementation lines and plant morphology study. Moreover, the recovery of the plant defect using these lines is an additional result to prove the epidermal defects is from the *mkp1* mutant. From these data, we can conclude MKP1 as a dual phosphatase that controls stomatal development within the plant epidermis. This phenotype was not only restricted to cotyledon epidermis, but also observed in other plant organs, such as the stem and rosette leaves.

4.2 No redundancy in function appeared using higher-order mutants

mkp1 stomatal phenotype indicates that this phosphatase partially regulates the activity of MPK3/6 due to the presence of mature stomata in the *mkp1* mutant. This mild phenotype led us to generate higher-ordered mutants based on *mkp1*. Unfortunately, at the double mutant stage, no significant difference was detected among these mutants regarding stomatal development. Meanwhile, no additive phenotype has showed up, however, in comparison to the *mkp1* stomatal phenotype. Higher-ordered mutant (triple mutant) generation is in progress to figure out if any redundancy in function could appear between these phosphatases during stomatal development. No redundancy in function between these phosphatases lead into a conclusion that MKP1 is the only dual phosphatase has an effect on the stomatal development.

4.3 Arrested stomatal lineage cells identity of *mkp1* mutant phenotype and suggested direction toward pavement cell.

TMM receptor-like gene is expressed in all stomatal-lineage cells. Using TMM as a stomatal-lineage cell marker, we identified the arrested cells in *mkp1* mutant as of stomatal-lineage origin. Moreover, tracking of *mkp1* cotyledons between 3 dpg and 11 dpg developmental stages has suggested the final destination of these arrested cells. During development of *mkp1* mutant plant, these arrested cells lost their stomatal-lineage cell identity and were destined to become pavement cells later on. To understand the switch of cell identity, further experiments need to be done in order to clarify the mechanisms of how stomatal origin cells converted toward another type of cells and to understand cell plasticity.

4.4 Phosphatase activity of MKP1 is essential for its function during stomatal development.

Single mutation in the catalytic domain is certain to inactivate the phosphatase activity of MKP1. Like all the other dual-specific phosphatases, the conversion of cysteine residue in the catalytic centre of MKP1 into another amino acid (serine) abolished MKP1 phosphatases activity. Previous study reported that the conserved catalytic center is essential for AtMKP1 to function during genotoxic stress signaling (Ulm *et al.,* 2002). Our study has further revealed the implication of MKP1 in stomatal development. Unrescued phenotype in *mkp1* expressing

transgene of *MKP1*^{C235S} is considered as a concrete evidence for the importance of this catalytic centre during stomata development.

4.5 *MKP1* epistatic location is upstream of MPK3/6 and downstream of YDA.

MAPK phosphatases activity is usually linked to the upstream signaling pathways. In this study, we have found that MKP1 is complying with the activity of YDA due to the epistatic location where MKP1 located downstream of YDA during stomatal development and whole plant growth. But this evidence has to be verified by looking to the phosphorylation of MKP1 in *yda* mutant. As mentioned before that *yda* mutant has clusters of stomata and die before its reproductive maturity. Crossing *yda* with *mkp1* generates normal plants without any growth defects, means that *mkp1* eliminates the lethal phenotype of *yda* which in turn eliminates *mkp1* morphological defects. What is interesting as known that the defected growth phenotype in *mkp1* is caused by SA accumulation but in the double mutant *mkp1yda* these growth alterations disappeared indicating that SA accumulation reduced somehow after *yda* mutation, suggesting that maybe YDA and MKP1 act as antagonistic regulators in the SA synthesis. Data need to be confirmed by studying the SA biosynthesis in *yda* mutant's plants.

During stomatal development and besides the proposed function of MPK3/MPK6 as a regulator of the polarity of asymmetric cell divisions, genetic study in this work revealed the epistatic of *mkp1* stomatal phenotype by the main kinases in MAPK pathway. This is another evidence confirming the involvement of MKP1 in this pathway during stomatal development and upstream to MPK3 and MPK6.

4.6 MKP1 regulates the activity of SPCH and SCRM via MPK3/6 dephosphorylation during stomatal development.

By specifying the upstream epistatic location of *MKP1* with respect to MPK3/6 as a substrate regarding stomatal development, we were able to clarify that the initial stage of stomatal development is the target of MKP1. Here, we propose a new model, where MKP1 dephosphorylates MPK3/6. Inactivated MPK3/6 are thus unable to phosphorylate SPCH, so no

degradation of this transcription factor and thus, continues entry for stomatal lineage development. (Figure 12).

This new model clarified the equivalence system were plants used to maintain the balance between stomata development and pavement cells. It means under the standard condition of growth, plants manage an optimal number of stomata by regulating the phosphorylation activity of MPK3/6, therefore, control the entrance or refrain from initiating stomatal development.

It is interesting how same MAPK cascade involved in different biological processes without any cross talk or mistakes. As a new model of stomata development (Figure 12), the involvement of the dual specificity phosphatase (MKP1) within this signaling pathway at the level of regulating MPK3/6 activity, somehow clarified the principles of MAPK signaling specificity, at the level of stomata development.

Figure 13.

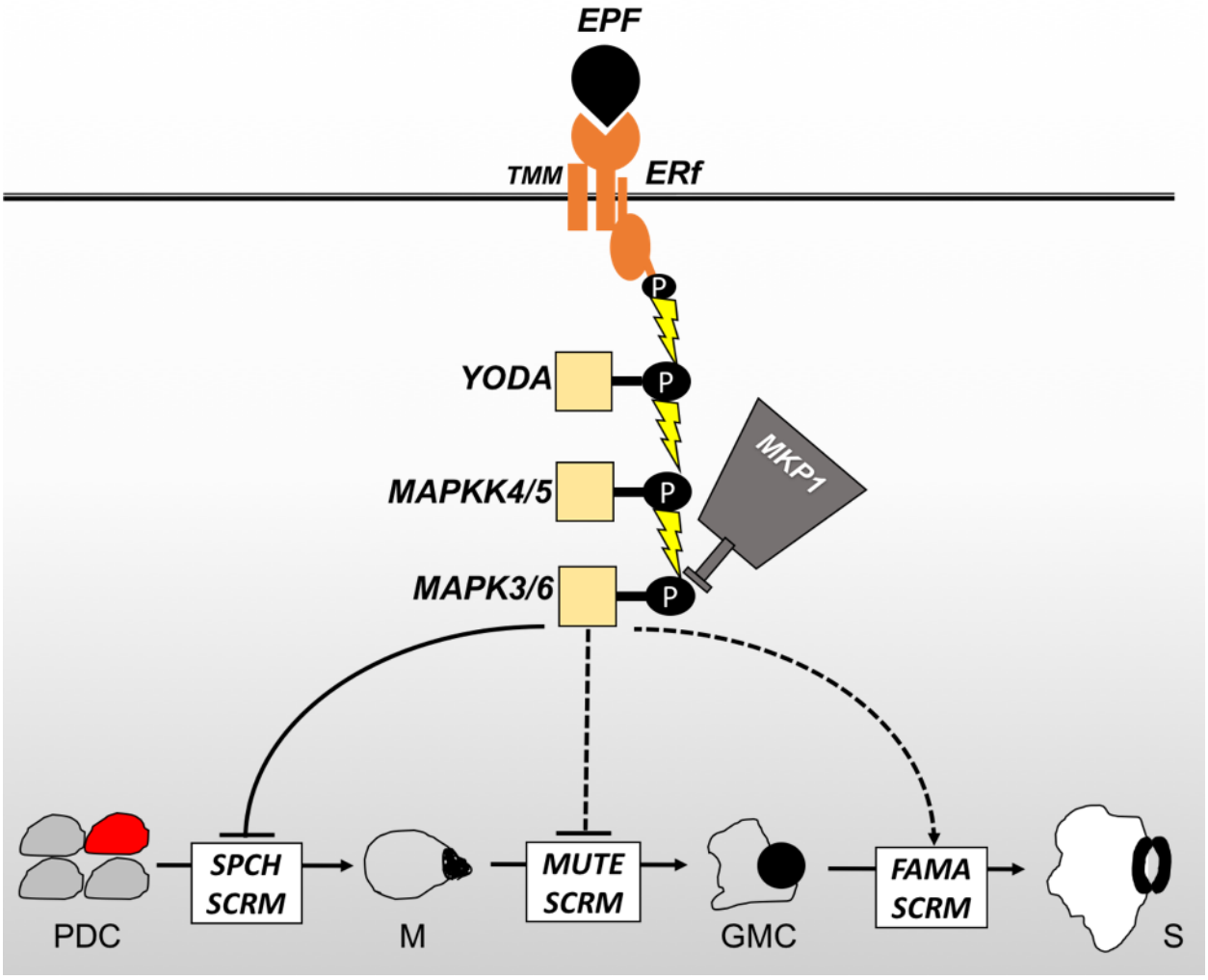
EPF
TMM
ERf
P
YODA
MAPKK4/5
MAPK3/6
MKP1
SPCH
SCRM
MUTE
SCRM
FAMA
SCRM
PDC
M
GMC
S

Figure 13. New diagram showing Signal pathway and its component with *MKP1* that regulate stomatal development

EPF ligands generate signalling via their interaction with TMM and the ERf receptors. Signal translated to YDA, MKKs then MPKs. MPK3/6 phosphorylate SPCH to inactivate it to reduce the initiation division. MKP1 phosphatase inhibits the activity of MPK3/6 at the initiation stage of stomata development. Predicted effects on MUTE and FAMA are labelled with dashed lines

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