SIGNALING EVENTS IN ARABIDOPSIS STOMATAL DEVELOPMENT AND PATTERNING

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A dissertation submitted to the

School of Graduate Studies
Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

 Graduate Program in Plant Biology

Written under the direction of

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New Brunswick, New Jersey

OCTOBER, 2019
Stomatal guard cells (GC) mainly exist in the epidermis of plant leaves and stems. They mediate the gas exchange between plants and the environment, thus are important for plant growth and development. In Arabidopsis, stomatal development and patterning are regulated by a linear signal pathway, mainly comprised of a set of peptide ligands, a suite of receptor-like protein/kinases, a mitogen-activated protein kinase (MAPK) cascade, and a series of transcriptional factors. Plant hormones and environmental factors regulate stomatal development through crosstalk with multiple components in this pathway. The MAPK cascade receives upstream signals from the cell surface to transduce to downstream intracellular factors, thus plays a central role in the regulation of stomatal development. The major goal of my Ph.D. thesis is to understand the signaling events occurring upstream and downstream of the MAPK cascade and how these signaling events are integrated to regulate stomatal development and patterning in Arabidopsis.
In the first chapter of my thesis, I identify the protein phosphatase 2A (PP2A) as new regulators in stomatal development and determine that they function antagonistically with MAPK3/6 and other kinases on the regulation of a master transcription factor in Arabidopsis. The PP2A phosphatase is a heterotrimeric complex, which is composed of three subunits (A, B, and C). In my studies, loss-of-function mutations in the genes encoding the subunits A and C both lead to suppressed stomatal production, suggesting the positive role of PP2A. The genetic analysis places PP2A function downstream of the MAPK cascade but upstream of the key transcription factor SPEECHLESS (SPCH). Pharmacological treatment supports the positive role of PP2A on SPCH protein stability. I further show that SPCH directly binds to PP2A-A subunits in vitro. Consistent with the hypothesis that PP2A dephosphorylates and stabilizes SPCH, in plants, the protein abundance of phospho-deficient SPCH appears less sensitive to the PP2A activities. Previously, multiple kinases, including MAPK3/6, BIN2, and CDKA;1, were identified to regulate SPCH protein phosphorylation that is related to protein stability and function, but it remained unknown which phosphatase combats these processes. Thus, my research identifies PP2A as the missing phosphatase that functions antagonistically with the known kinases to balance the phosphorylation status of the master transcription factor SPCH in Arabidopsis stomatal development. This work is currently under view at PNAS, on which I am first-authored.

In the second chapter of my thesis, the research investigates the D6 protein kinase (D6PK) for their functional connection with cell polarity in the regulation of stomatal
development and patterning. This work was initiated from the collaboration with Dr. Claus Schewechheimer (TUM, Germany) and conducted by myself and the collaborative effort of Dr. Kezhen Yang (visiting scholar in our lab). We provide genetic evidence that D6PK regulates stomatal development and patterning by participating in the BASL-YDA polarity complex in Arabidopsis stomatal lineage cells.

BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) is a plant-specific protein, which has a unique polarized localization at the cell cortex. The loss-of-function mutant of BASL shows clustered stomata and abnormal cell division pattern. A recent study indicated phosphorylated BASL recruits the MAPKKK YODA (YDA) and MPK3/6 to the cell cortex to active the MAPK cassette to inhibit stomatal cell fate. However, what other components in the polarity complex and how the BASL-YDA module is regulated by other genetic components remain largely unknown.

In this study, we report the D6PK family as a new regulator in stomatal development and patterning. I show that the D6PK family loss-of-function mutants and the overexpression of D6PK both generate stomatal defects in Arabidopsis. In the stomatal lineage cells, we found that D6PK associates with BASL-YDA polarity complex at the cell cortex, and D6PK polarity is dependent on the presence of BASL. Further, our genetic analysis suggests D6PK functions upstream of the BASL-YDA complex, and D6PK possibly regulates BASL protein stability. Thus, our data suggest the D6PK protein family regulates stomatal development and patterning by participating and regulating the BASL-YDA complex.

The last chapter of my thesis is achieved by my collaborative effort with Dr. Xueyi Xue
(Post-doctoral fellow in the lab). Here, we identify and characterize a new family of MAPK substrates for their function in Arabidopsis stomatal development. The MAPK cascades play critical roles in many aspects in plant growth, development and stress responses. Stomatal development and patterning in Arabidopsis are tightly regulated by a canonical MAPK signaling cascade composed of the MAPKK kinase YODA, MAPK Kinase 4 and 5 (M KK4/5) and MAPK 3 and 6 (MPK3/6). Three MAPK SUBSTRATES IN THE STOMATAL LINEAGE (MASS) proteins were isolated from a large-scale screen of substrate peptides modified by Arabidopsis MPK3 and MPK6. The loss-of-function and overexpression phenotypes suggest MASS functions as a positive regulator of stomatal production in Arabidopsis. Furthermore, we provide experimental evidence supporting that MAPK-mediated phosphorylation is important for the subcellular localization and function of MASS, in turn, the MASS proteins interact with YDA, likely to suppress YDA’s function at the plasma membrane. Thus, the functional connection between the MASS family and the YDA MAPK cascade represent a negative feedback loop, providing a mechanism to diverge the MAPK functional output in the regulation of stomatal development. The manuscript was submitted to PLOS Genetics, on which I am a co-first author.
ACKNOWLEDGEMENTS

First, I would like to express my sincere thanks to my advisor Dr. Juan Dong. In the past six years, her support and advice helped me to grow as a scientist and to become a better person. She trusted me and gave me lots of opportunities to prove and improve myself. I would also like to thank every current and former member in our lab. Particularly, Dr. Kezhen Yang as a visiting scholar in our lab provided me lots of valuable suggestions in project design and biochemical assays. Dr. Xueyi Xue gave me lots of help with molecular cloning and genetic analysis. Dr. Xiaoyu Guo gave me tremendous help with biochemical experiments. Dr. Yi Zhang and Lu Wang also gave me valuable suggestions on my projects. Thanks for being not just great advisors and colleagues, but families and friends.

My sincere thanks also go to Dr. Andrea Gallavotti, Dr. Pal Maliga, and Dr. Christopher Rongo for serving as my committee members. Their valuable comments and suggestions inspired me to widen my research from various perspectives.

Special thanks to my family: my parents, my sister and my wife, for their unqualified support. They mean everything to me. I am so lucky and blessed to have them.

Last but not least, I would like to thank all the people who provided me help in the past six years, the Waksman Genomics Core Facility, and the fellowships supported my study, including China Scholarship Council (CSC) fellowship, the Charles and Johanna Busch Predoctoral Fellowship, and the Teaching Assistantship from Rutgers University.
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CHAPTER ONE
INTRODUCTION

Arabidopsis thaliana is a small flowering plant that belongs to the mustard (Brassicaceae) family, which includes cultivated species such as cabbage and radish. Arabidopsis as a widely used model system in plant biology offers important advantages for basic research in genetics and molecular biology, such as relatively small genome size, rapid life cycle, prolific seed production, and efficient transformation method. Stomata are guard cell (GC) pores mainly on the surface of aboveground organs that mediate gas exchange between plant and the atmosphere (1). They allow plants to intake carbon dioxide for photosynthesis while limiting water loss from plants. This balance can be achieved by controlling the opening and closing of GCs, and alternatively through regulation on stomatal proliferation and distribution in the epidermis (2).

Overview of stomatal development in Arabidopsis

In Arabidopsis, the formation of stomata involves cell divisions and cell fate differentiation of a specialized epidermal cell lineage, the stomatal lineage (2-4). There are five major cell types of stomatal lineages, including meristemoid mother cell (MMC), meristemoid (M), stomatal lineage ground cell (SLGC), guard mother cell (GMC), and GC. The MMC cell originates from a protodermal cell, although the selection process is not well understood. The MMC undergoes an asymmetric division and forms a larger daughter cell (SLGC), with a smaller daughter cell meristemoid (M) that usually has triangle cell shape. This division is also known as entry division. The
M may undergo additional asymmetric divisions and create several new Ms and new SLGCs; this process is also called the amplifying division. The M eventually differentiates to the GMC after undergoing several rounds of amplifying divisions. Meanwhile, SLGCs can also carry out additional asymmetric divisions to create satellite Ms (spacing division), and eventually differentiate into pavement cells. The precursors of GCs, GMCs are small cells usually with rounded cell shape; they undergo a single symmetric division to form a pair of GCs. In Arabidopsis, stomata are distributed across the leaf area in diverse patterns and following the ‘one-celled spacing’ rule, which posits that stomata have at least one pavement cell between them (5). This spacing pattern optimizes the efficiency of gas exchange (6).

**The bHLH proteins regulate stomatal cell fate transitions**

In the stomatal lineage, there are three closely related basic helix-loop-helix (bHLH) transcription factors, SPEECHLESS (SPCH), MUTE, and FAMA, sequentially expressed in specific cell types to promote cell fate transitions (7-9). SPCH is expressed the earliest in stomatal development, mainly in MMCs and Ms, and it is required to initiate the MMCs and subsequent asymmetric divisions (7, 8). In the loss-of-function *spch* mutant, the stomatal initiation is completely suppressed, so the epidermis is only comprised of pavement cells (7). MUTE is mainly expressed in Ms and it terminates stem cell behavior by promoting the Ms differentiation into GMCs (7, 8). In the loss-of-function mutant of MUTE, the stomatal lineages are initiated but the differentiation of Ms is totally inhibited, so all Ms are arrested and no mature GCs are formed in the epidermis. Meanwhile, constitutive overexpression of MUTE produces...
an epidermis almost occupied by only GCs, including some cells transdifferentiated from pavement cells (7, 8). The bHLH FAMA is expressed in GMCs and young GCs, and it drives the terminal differentiation from GMCs to GCs (9). The loss-of-function mutant of FAMA completely lacks mature GCs but instead produces clusters of small and narrow tumor cells in the epidermis. The overexpression of FAMA also converts non-stomatal cells to GCs, but these GCs are not properly paired (9). Different from MUTE and FAMA, the overexpression of SPCH does not produce extra stomata, suggesting the post-translational regulation on SPCH protein in plants (7). Besides, FAMA recruits and interacts with the cell cycle regulator Arabidopsis Retinoblastoma homolog, RETINOBLASTOMA-RELATED (RBR) to suppress SPCH and other stomatal genes, thus promoting the irreversible terminal differentiation of GCs (10). Besides of these three bHLH transcriptional factors, two related bHLH-leucine zipper (bHLH-LZ) proteins, INDUCER OF CBF EXPRESSION 1 [ICE1, also known as SCREAM (SCRM)] and SCRM2, were identified as partners of SPCH, MUTE, and FAMA (11). The double mutant of loss-of-function ICE1/SCRM and SCRM2 resembles spch mutant, lacking stomata in the epidermis. The gain-of-function ICE1/SCRM allele also generates an epidermis entirely composed of stomata. ICE1/SCRM, SCRM2 and the three bHLH transcription factors probably function as heterodimers in binding and regulating the downstream targets (11). A recent study also suggested ICE1/SCRM functions as a scaffold that recruits the mitogen-activated protein kinase 3/6 (MAPK3/6) to downregulate SPCH function in stomatal development (12).
Signaling components and pathways in the regulation of stomatal development and patterning

After almost two decades of study, a relative linear genetic pathway was identified to regulate the stomatal development and patterning, including secreted peptide ligands, leucine-rich repeat receptor kinases (LRR-RKs) at the plasma membrane, intracellular mitogen-activated protein kinase (MAPK) signaling transduction cascade, and the master bHLH transcription factor SPCH (1-3). Receptor proteins at plasma membrane perceive the signal from peptide ligands outside of cell wall, then MAPK cascade is activated, leading to phosphorylation of SPCH attenuating its activity and stability.

Fig. 1. Stomatal development and patterning in Arabidopsis
Stomatal formation requires an asymmetric cell division of an MMC (grey) and formation of the meristemoid (red), the differentiation of meristemoid to GMC (orange), and the symmetric cell division of GMC to form a pair of GC (green). The confocal
image of a cotyledon illustrates stomatal lineage cell types in Arabidopsis. Stomatal formation and patterning are regulated by a genetic pathway, including the peptide ligands (EPFs), receptor proteins (ERFs and TMM), MAPK cascade, the BASL-YDA-MPK3/6 positive feedback loop, and a series of transcription factors.

- **The peptide ligands**

Stomatal development and patterning are controlled by several key peptides in Arabidopsis, including EPIDERMAL PATTERNING FACTORS 1 and 2 (EPF1, and EPF2), STOMAGEN (also referred to EPIDERMAL PATTERNING FACTOR LIKE 9, EPFL9), and CHALLAH-related family (CHALf) (15-19). The functions of these peptides are divergent. EPF1/2 negatively regulate stomatal development by binding to and activating the LRR-RKs, while the STOMAGEN is a positive regulator that competes for binding of the LRR-RKs and associated proteins with EPF1/2 (20, 21). The functions of EPF1 and EPF2 are similar (22). EPF2 is expressed earlier than EPF1, and it predominately regulates SPCH function in stomatal development; while EPF1 mainly regulates stomatal patterning by enforcing the ‘one-celled spacing’ rule in stomatal lineages (22, 23). The loss-of-function mutant of EPF1 leads to breaking of the ‘one-celled spacing’ rule and stomatal clustering (two or more stomata in contact with each other). The epf2 mutant exhibits excessive cell divisions and increased cell density (the number of stomata per leaf area) (15, 16). Overexpression of EPF1 and EPF2 both decreases stomatal production in the epidermis (15, 16). In contrast with EPF1/2, which are expressed in stomatal lineage cells, STOMAGEN is expressed in mesophyll cells, suggesting its non-autonomous function in stomatal lineages. Suppressed gene function of STOMAGEN leads to a reduction of stomatal production, while overexpression of STOMAGEN drastically increases stomatal
density and causes stomatal clusters (17, 18). Another subgroup of EPFL family, the CHALf includes three paralogs that are expressed in internal layers of hypocotyls and stems, CHAL (EPFL6), CHALLAH-LIKE1 (CLL1, EPFL5), and CLL2 (EPFL4). They are perceived by LRR-RKs and their functions are inhibited by the non-kinase receptor TOO MANY MOUTHS (TMM) (19, 24).

- **The receptors at the plasma membrane**

  Plant genomes encode large families of LRR-RKs and the associated LRR-receptor-like proteins (LRR-RLPs). LRR-RKs are the largest subfamily of transmembrane receptor-like kinases in Arabidopsis and they are involved in a wide range of plant developmental processes (25, 26). The associated LRR-RLPs modulate the function of LRR-RKs by providing cellular and tissue specificity.

  One of the most studied LRR-RKs, the ERECTA family (ERf), composed by ERECTA (ER), ERECTA-LIKE 1 (ERL1) and ERECTA-LIKE 2 (ERL2), is important for many aspects of plant growth, including leaf, floral, root, vascular and stomatal development (27). In stomatal lineages, these three ERf members redundantly regulate stomatal development and patterning, and ER is the dominant player (28). As knocking-out more genes in ERf, the stomatal defects are more severe. The er;erl1;erl2 triple mutant leads to dramatically increased stomatal production and stomatal clusters (28).

  An LRR-RLP protein TMM is also required for stomatal development and patterning (29). TMM lacks a C-terminal kinase domain and provides specificity to ERf by forming heterodimeric receptor complexes with them. TMM is specifically expressed in stomatal lineages and it seems to function paradoxically in leaves and hypocotyls.
The loss-of-function *tm* mutant exhibits enhanced stomatal density and stomatal cluster in cotyledons but produces no stomata in hypocotyls (29). A recent study revealed that the tissue-specific expression of LRR-RKs in conjunction with TMM could create precise grooves to interact with different EPF peptide ligands (30). Recent studies showed another LRR-RK family, the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family is involved in stomatal development, plant immunity, programmed cell death regulation and Brassinosteroid (BR) signaling (31-33). There are five homologs (SERK1 to SERK 5) in Arabidopsis, and genetic evidence showed SERK3 (also known as BAK1, BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1), SERK2, SERK1, and SERK4 redundantly act as co-receptors for peptide signaling in stomatal development (31). The quadruple serk mutant showed a similar level of stomatal production and clustering as *er;er1;er2* triple mutant. The SERKs form complexes with ERf members and TMM to transduce the peptide signals to downstream MAPK cascade (31).

- **The cytoplasmic MAPK signaling cascade**

Once cytosolic kinase domains of the ER/SERK/TMM complexes become phosphorylated, it triggers a series of downstream phosphorylation events, including the MAPK cascade (14, 34-36). Plant MAPK cascade, like in yeast and animal systems, are used to mediate developmental, stress and defense responses. It includes the sequential phosphorylation and activation of a three-kinase module: the MAPK kinase kinases (MAPKKKs), the MAPK kinases (MKKs) and the MAPKs (MPKs) (37). In stomatal development, there are at least eight kinases: the MAPKKK
YODA (YDA), MKK4, MKK5, MKK7, MKK9, MPK3, MPK4 and MPK6 (34, 35, 38). The previous genetic analysis placed the MAPK cascade downstream of ERf signaling, and biochemical evidence showed MPK3 and MPK6 directly phosphorylate and suppress bHLH SPCH function (14). Loss-of-function *yda* mutant produces excessive and clustered stomata but are often embryo lethal and defective in several other developmental and physiological responses. ΔN- YDA, a constitutively active form of YDA generates a phenotype similar to that of an *spch* mutant, with no stomata formation in the epidermis (34). After YDA is activated by upstream receptor signaling, it phosphorylates the next class of proteins in MAPK cascade, the MKKs. Among 20 MKKs in Arabidopsis, at least four of them (MKK4/5/7/9) are involved in stomatal development. They are able to repress the transition of M to GMC (35, 36). The final step in MAPK cascade is the phosphorylation of MAPKs by MKKs. Co-immunoprecipitation assays showed direct interactions between MPK3/6 and SPCH, but not MUTE and FAMA (36). MPK4 was also shown to phosphorylate MUTE in vitro, but there is no in vivo or phenotypic evidence to confirm the phosphorylation of MUTE (36). Loss-of-function MKK4/MKK5 or MPK3/MPK6 disrupts the coordinated cell fate specification of stomata versus pavement cells, resulting in the formation of excessive and clustered stomata. Conversely, activation of MKK4/MKK5 or MPK3/MPK6 causes the suppression of asymmetric cell divisions and stomatal cell fate specification, resulting in a lack of stomatal differentiation (36).

- **Regulation of SPCH by protein phosphorylation**
The peptide signaling acting through LRR-RKs and MAPK cascade functions to phosphorylate SPCH, and affect its protein stability and activity in stomatal development (3). Different from the other two bHLH transcription factors MUTE and FAMA, SPCH protein harbors a specific MAPK-targeting domain (MPKTD) that is regulated by MAPK phosphorylation (3). Site-directed mutagenesis study revealed the function of several potential phosphorylation sites in controlling SPCH function. SPCH variants with all five phosphorylation sites mutated to alanine (SPCH^{1-5A}) promote the transition of Ms to GMCs, while the SPCH^{1-4A} variants that only the first four phosphorylation sites are mutated to alanine promote stomatal asymmetric divisions. Deletion of the entire MPKTD in SPCH (SPCH^{Δ93}) leads to excessive stomata clusters (3).

Besides of MAPKs, an important signaling intermediate, the glycogen synthase kinase3 (GSK3)-like kinase, BR INSENSITIVE 2 (BIN2), was also shown to phosphorylate SPCH protein and regulate its protein stability (4). These phosphorylation residues overlap with those targeted by MAPKs, as well as five residues in the N-terminal of SPCH that is outside of the MPKTD. The SPCH variant with these five phosphorylation sites mutated to alanine (SPCH_{S/T38-44A-S65A}), similar to inhibition of BIN2 activity in vivo, both stabilize SPCH protein function and triggers asymmetric cell divisions in the epidermis (4).

The CYCLIN-DEPENDANT KINASE A;1 (CDKA;1), an important component in cell cycle regulation pathway, also phosphorylates SPCH and regulates its activity (5). Biochemical assays showed CDKA;1 phosphorylates SPCH at serine 186. The SPCH
variant SPCH$^{S186A}$ is not able to complement the spch null allele, indicating the phosphorylation of Serine 186 is essential for SPCH function. The cdka;1 mutant has undetectable levels of SPCH protein and produces no stomata in the epidermis, suggesting the phosphorylation of SPCH by CDKA;1 promotes its protein stability and promotes stomatal development (5).

The polarity protein BASL and the crosstalk with the MAPK signaling cascade

In stomatal lineages, a polarity protein, BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL), has been shown to function at cell cortex to regulate the activity of MAPK cascade (6). The loss-of-function mutant of BASL breaks the ‘one-celled spacing’ rule and exhibits stomatal clusters as well as excessive small cell divisions. In stomatal development, during the entry division that forming an M and SLGC from an MMC, SPCH expression is normally maintained in the M but degraded in the SLGC by MPK3/6. In contrast, BASL is expressed in the nucleus of Ms and polarly localized at the plasma membrane in SLGCs (7, 8). The phosphorylation of BASL by MPK3/6 promotes BASL accumulation at the polarity site in SLGCs, and it further recruits components in MAPK cascade at the polarity site, including YDA and MPK3/6 and enriches the MAPK signaling in SLGCs. The SPCH protein is therefore phosphorylated by MPK3/6 and its stability is suppressed (7, 8). Besides, a recent study suggested another plant-specific protein POLAR, together with BASL, function as a stomatal scaffold and recruit GSK3-like kinases to the polarity site before the asymmetric cell division. As a result, the MAPK signaling is attenuated, enabling SPCH to drive asymmetric cell divisions in the nucleus. Moreover, BIN2 initiates
BASL polarization redundantly with the MAPK signaling, and the turnover of POLAR requires BIN2 phosphorylation (9).

**Hormonal and environmental factors in the regulation of stomatal development**

Recent studies have revealed that, besides the intrinsic signals, some other signals also participate in regulating stomatal development and patterning, including plant hormones and environmental factors (10).

BR is a plant steroid hormone that regulates diverse growth and developmental processes. An important signaling intermediate, BIN2 regulates stomatal development at multiple levels. As mentioned above, BIN2 phosphorylates and suppresses the bHLH SPCH (negative effect). Besides, BIN2 also directly phosphorylates and inactivates the MAPKKK YDA (therefore positively regulates stomatal development) (4, 11). These results reveal a discrepant role of BR in regulating stomatal development, suggesting a fine-tuning mechanism for targeting SPCH during its successive steps of activities. Inactivation of BIN2 protein family suppresses stomatal production, and the quadruple mutant of BIN2’s direct upstream inhibitor (the BSU1 phosphatase family) produces an epidermis composed mostly of stomata clusters, similar to the yda mutant (11).

Auxin is an important plant hormone that broadly regulates various plant developmental processes, and its role in stomatal development and patterning was reported recently (12-14). The time-lapse experiment indicates that auxin activity changes over stomatal development. The depletion of auxin from Ms is required for the transition from Ms to GMCs, probably via the auxin efflux transporter PIN
The auxin signaling is also involved in stomatal development. Auxin is perceived by the nuclear receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN-BINDING F-BOX (AFB), which binds to AUXIN/INDOLEACETIC ACID (Aux/IAA) proteins. The subsequent degradation of Aux/IAA releases its suppression on transcription factors AUXIN RESPONSE FACTORs (ARFs), thus auxin response is activated (15). Auxin plays a negative role in stomatal development, since exogenous application or genetic manipulation of auxin reduces the number of stomata and lineage cells, while auxin-deficient, auxin transport-deficient, or auxin signaling-deficient mutants produce stomatal clusters (12-14). Consistently, stabilizing the suppressor Aux/IAA AUXIN RESISTANT3 (AXR3/IAA17) and loss-of-function of MONOPTEROS (MP)/ARF5 all increase stomatal formation (13, 14). Further evidence showed MP/ARF5 binds to the promoter of STOMAGEN and regulates its expression. The target of AXR3 remains unknown, and the inhibition effect on stomatal development mediated by AXR3 only occurs in dark, suggesting the crosstalk with light signaling (13).

Light is one of the environmental signals that dramatically affect stomatal development (16-19). Light signals are perceived by photoreceptors and then transduced to suppress the RING E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) activity (20). Plants with mutations in these photoreceptors show reduced stomatal density. Loss-of-function mutants of COP1, as well as mutants of other COP genes, such as COP10, generate stomatal clusters (19). Recent studies identify the bHLH proteins ICE1/SCR and SCR2 as targets for
COP1-mediated degradation in dark, thus preventing the formation of stomata in etiolated seedlings (21).

Carbon dioxide (CO$_2$) is a source of plant photosynthesis and absorbed through stomata. Recent studies revealed that elevated concentrations of CO$_2$ reduce the stomatal density, indicating its negative role in stomatal development (22). This repression of stomatal formation by elevated CO$_2$ probably requires EPF2-mediated signaling pathway, because this repression is converted by mutation of $EPF2$ (23).

Besides, water condition is also known to affect stomatal development, although the detailed mechanism is not well understood. Low water potential may be generated by less water or high osmotic pressure. In grasses, drought stress reduced stomatal density; and in Arabidopsis, high osmotic pressure results in fewer stomata formation due to the destabilization of SPCH protein (24-26). Overexpression of $EPF2$ and $EPF1$ reduced stomatal formation and improved drought tolerance in Arabidopsis and barley, respectively (27-29). Further studies are required to better understand how plants fine-tune stomatal formation in response to different water conditions.

**Research topics in my Ph.D. study**

Plant stomata mediate the gas and water exchange between plants and atmosphere thus is important for plant growth and development. In Arabidopsis, stomatal development and patterning are controlled by multiple factors, including external peptide ligands, intrinsic polarity signals, plant hormones, and environmental factors. The MAPK signaling cascade and the downstream transcription factors are usually recruited by different signaling pathways to participate in stomatal development and
patterning. How these signaling pathways are integrated and coordinated to control functions of MAPK cascade and these transcription factors will be interesting and important topics. Within this context, there are several specific questions need to be answered to better understand the mechanism.

- The key transcription factor SPCH is post-translationally regulated by multiple kinases through phosphorylation, and the phosphorylation of SPCH leads to protein degradation and/or activity change. However, how these kinases coordinate to balance the phosphorylation level of SPCH and maintain its proper function in stomatal production remains poorly understood. Meanwhile, what phosphatases regulate the de-phosphorylation of SPCH is unknown. In chapter two, we determine that the phosphatase PP2A promotes SPCH protein stability by direct interaction via the A subunits.

- The intrinsic polarity protein BASL and external peptide signaling both regulate stomatal patterning, but how these two distinct pathways coordinate to ensure the correct stomatal patterning was very poorly understood. Previous studies suggest both signaling pathways may recruit the MAPK cascade to pass signal to downstream components. It would be interesting to test if there is any interaction between these two genetic pathways in stomatal context. In chapter three, we describe the AGC VIII kinase D6PK family controls stomatal development and patterning, possibly participate in BASL-YDA polarity complex and other genetic pathways.
MAPK signaling cascade targets a number of substrates to transduce the upstream signals. Among these MAPK substrates, the bHLH SPCH may be the most important target in stomatal regulation. However, SPCH mainly controls the stomatal entry division and not participate in later differentiation stages. What other unknown substrates are targeted by MAPKs in the regulation of stomatal development and patterning is an important research topic. In chapter four, we report new MAPK substrates, the MASS protein family that regulates stomatal development and patterning by forming a negative feedback loop with the MAPK cascade.

In summary, my thesis describes several signaling events in the MAPK cascade or its downstream tier and how these events assist our understanding of the stomatal development and patterning mechanisms.
REFERENCES FOR INTRODUCTION


CHAPTER TWO

Protein Phosphatase 2A promotes stomatal development by stabilizing SPEECHLESS in Arabidopsis

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This chapter is in the format of the manuscript submitted to the PNAS, and A. DeLong provided valuable suggestions in the manuscript preparation.
Abstract

Stomatal guard cells control gas exchange that allows plant photosynthesis but limits water loss from plants to the environment. In Arabidopsis, stomatal development is mainly controlled by a signaling pathway comprised of peptide ligands, membrane receptors, a mitogen-activated protein kinase (MAPK) cascade, and a set of transcriptional factors. The initiation of the stomatal lineage requires the activity of the bHLH transcriptional factor SPEECHLESS (SPCH) with its partners. Multiple kinases were found to regulate SPCH protein stability and function through phosphorylation, yet no antagonistic protein phosphatase activities have been identified. Here, we establish the conserved PP2A phosphatases as positive regulators of Arabidopsis stomatal development. We show that mutations in genes encoding PP2A subunits result in lowered stomatal production in Arabidopsis. Genetic analyses place the PP2A function downstream of the MAPK cascade, but upstream of SPCH. Pharmacological treatments support a role for PP2A in promoting SPCH protein stability. We further show that SPCH directly binds to the PP2A-A subunits in vitro. In plants, non-phosphorylatable SPCH proteins are less affected by PP2A activity levels. Thus, our research identifies PP2A as the missing phosphatases that function antagonistically with the known kinases to maintain the phosphorylation balance of the master regulator SPCH in stomatal development.
Significance

The innovation of stomatal pores in plant evolution enables the success and diversification of land plants over the past 400 million years. The production and patterning of stomata are regulated by a group of deeply conserved bHLH transcription factors in land plants, with the founding member SPEECHLESS (SPCH) playing a pivotal role in the initiation of stomatal lineage. The protein stability and function of SPCH are tightly regulated by protein phosphorylation mediated by multiple kinases in Arabidopsis. Here, we establish the highly conserved PP2A protein phosphatases as positive regulators of SPCH and thus stomatal production, underscoring a key function of PP2A in fine-tuning stomatal development, a highly plastic biological process that influences many plant responses to changing environmental conditions.

Introduction

Stomata are epidermal pores, each of which is girdled by a pair of guard cells that control stomatal opening and closing to mediate gas exchange between plants and the atmosphere. The formation of stomata involves stereotypic cell divisions and cell fate differentiation of the stomatal lineage cells, all of which are tightly controlled by a suite of closely-related and sequentially-expressed basic helix-loop-helix (bHLH) transcriptional factors in the model plant Arabidopsis thaliana. The bHLH SPEECHLESS (SPCH) acts earliest and is mainly expressed in meristemoid mother cells (MMCs) that divide to produce meristemoids (Ms) and stomatal lineage ground
cells (SLGCs). SPCH is required to initiate the MMCs, and thus the formation of stomata, in the epidermis of a plant (1, 2). The differential developmental trajectories of the Ms and the SLGCs coordinate the production of stomatal guard cells and pavement cells in a leaf. The closely related bHLH MUTE is mainly expressed in late Ms to promotes cell fate transition to guard mother cell (GMCs) (1, 2). Another related bHLH FAMA is expressed in GMCs and young guard cells (GC), driving GMC terminal differentiation into GCs (3). In addition, two bHLH-leucine zipper (bHLH-LZ) proteins, INDUCER OF CBF EXPRESSION 1 (ICE1, also known as SCREAM) and SCREAM2 (SCRM2) function to promote the three distinct sequential phases of differentiation in stomatal development by acting as partners of SPCH, MUTE, and FAMA (4). Among these bHLH transcription factors, SPCH appears to be a master regulator that directly drives the expression of other bHLH factors, e.g. MUTE, SCRM/ICE1, and SCRM2 (5).

Upstream of the core bHLH functional module, both environmental and developmental signals regulate stomatal development, most, if not all, of which eventually feed into the regulation of SPCH expression. In a well-established linear signaling pathway, the extracellular peptide ligands, EPIDERMAL PATTERNING FACTORs (EPFs), are perceived by receptors at the plasma membrane, e.g. the receptor-like protein TOO MANY MOUTHS (TMM), the receptor-like kinase ERECTA and the SOMATIC EMBRYOGENESIS RELATED KINASE (SERK) families (6-10). The ligand-receptor signaling is then transduced to a mitogen-activated protein kinase (MAPK) cascade, including MAPKKK YODA (YDA), MKK4/5, and MAPK3/6 (11, 12),
to trigger SPCH phosphorylation and protein degradation (13). The plant hormone brassinosteroid (BR) may promote stomatal production in the hypocotyl by suppressing a negative regulator of SPCH, the serine/threonine glycogen synthase kinase 3 (GSK3)/SHAGGY-like BRASSINOSTEROID insensitive 2 (BIN2), which phosphorylates SPCH for degradation (14). The cell-cycle regulator CYCLIN-DEPENDENT KINASE A;1 (CDKA;1) also phosphorylates SPCH but positively affects SPCH function via an unknown mechanism (15). Protein phosphorylation is highly dynamic and rapid phosphorylation-dephosphorylation cycles underlie many critical biological responses. The identity of the phosphatase[s] that counteract these kinases in the regulation of SPCH stability and function in stomatal development remains a long-standing question.

The conserved Protein Phosphatase 2A (PP2A) family of ubiquitously expressed Ser/Thr protein phosphatases in eukaryotes are heterotrimeric complexes that comprise a catalytic subunit C, a regulatory subunit B, and a scaffolding subunit A (16). The Arabidopsis genome encodes multiple isoforms for each subunit (3 As, 17 Bs, and 5 Cs) (17) (Fig. 1A), and differentially assembled PP2A complexes regulate plant growth, development, metabolism, and stress responses (18, 19). Functions of PP2A have been linked to the regulation of blue light-triggered stomatal movement by inhibiting the kinase activity of phototropin PHOT2 (20). Also, by associating with the SnRK2-type kinases, PP2A also participates in ABA-mediated stomatal closure (21). In addition, the determination of cell division orientation in maize stomatal complexes and in Arabidopsis embryos and seedlings requires PP2A activities in the formation of
the pre-prophase band (22, 23). Here, we establish a new function of PP2A in stomatal development: PP2A phosphatases positively regulate SPCH protein stability to promote stomatal production. The physical association of PP2A with SPCH is likely mediated by direct interactions between SPCH and the A subunits. Thus, our research revealed a missing regulator that balances the SPCH phosphorylation-dephosphorylation homeostasis in stomatal development. In addition, the PP2A components, as newly identified signaling molecules in the regulation of stomatal production, will be candidates to be manipulated in the future to fine tune stomatal development for plants to better adjust to unfavorable environmental conditions.

Results

The PP2A-A Mutants are Defective in Stomatal Production.

It has been previously reported that PP2A plays pivotal roles in plant development and growth patterning. Mutations in PP2A components led to defects in many developmental processes in Arabidopsis, such as inflorescence development (24), root elongation (24, 25), pavement cell morphogenesis (26) and cell division patterns (23). The Arabidopsis genome encodes three A subunits that are highly similar in their sequences (A2/PDF1 and A3/PDF2 are 94% identical and A2/PDF1 is 86% identical to A1/RCN1, shortened to A1, A2 and A3 in Fig. 5A and throughout this manuscript) (24), and we observed all three A subunit genes to be ubiquitously expressed in leaves (Fig. 5H-J). We examined the developmentally defective PP2A-A T-DNA
insertion mutants (24, 27) for possible defects in stomatal development, and detected obvious defects in some of these mutants. For example, both \( a_1; a_2 \) and \( a_1; a_3 \) mutants produced fewer stomata, measured by Stomatal Index (SI, number of stomata as a percentage of the total number of epidermal cells in a given area). The SI of \( a_1; a_2 \) and \( a_1; a_3 \) mutant plants was reduced to 10.7% and 12.9% (\( n=930 \) and 1844 total number of cells), respectively, compared to 23.7% in wild-type (WT) plants (Fig. 1B-D and quantification in Fig. 1J). The other double mutant \( a_2; a_3 \), which did not show overall growth defects, produced normal leaves with typical stomatal patterning (Fig. 1J and Fig. 5D), suggesting the important contribution of \( A_1 \) in stomatal development. Indeed, mutating \( A_1 \) alone already reduced stomatal production in plants (Fig. 1J and Fig. 5B, C, 19.8%, \( n=2445 \) vs. 23.7% in WT, \( n=1628 \)), while neither the \( a_2 \) nor \( a_3 \) mutation alone obviously changed stomatal and other developmental processes. We also crossed the double mutants \( a_1; a_2 \) and \( a_1; a_3 \), but failed to obtain homozygous triple mutants, suggesting that some \( A \) subunit function is essential for plant early development.

To further investigate the collective roles of the three \( A \) subunits in the regulation of stomatal development, we deployed CRISPR/Cas9-mediated mutagenesis (28) to simultaneously knock them out. Based on the high sequence similarity among the three PP2A-A subunits (24), we designed two guide RNAs (sgRNA1 and sgRNA2) that specifically target \( A_1 \) and \( A_2/A_3 \), respectively (Fig. 5A). Simultaneously introducing the two sgRNAs with Cas9 into Arabidopsis plants was anticipated to give rise to mutations in all three \( A \) genes (Fig. 5A). Indeed, we noted that the first
generation of transgenic plants (T1s) already showed growth defects, including
dwarfed stature (Fig. 5K) and inhibited root growth, as previously reported (24). Two
independent lines (crispr-a1;a2;a3 #7, and #12) were selected for thorough genotype
and phenotype analyses. We found that the mutants show greatly reduced SI, (Fig.
1E-G, and quantified in Fig. 1J, 2.4% in crispr-a1;a2;a3 #7, n=2183 vs. 23.7% in WT,
n=1628). Genotyping data suggested that these individual mutant plants were not
homogenous knockouts, but instead carry various somatic mutations. Specifically, the
plant crispr-a1;a2;a3 #12 (Fig. 5K) carried 1-bp insertions in A1 and A3 and a 6-bp
deletion in A2 (Fig. 5L). On the other hand, the crispr-a1;a2;a3 #7 plant is chimeric,
harboring a 1-bp deletion in A3 and multiple mutations in A1 and A2 (shown in Fig.
5L). We were unable to obtain any Cas9-free mutants from the plants that showed
strong stomatal phenotypes, hinting that the loss-of-function mutations in all three
PP2A-A genes might be detrimental to gametophyte and/or embryo development.

To further verify the stomatal phenotype we observed, we generated knockdown
plants using the miRNA-induced gene silencing strategy (29). Specifically, a highly
conserved coding region in PP2A-A2 (93% identical to A3, and 81% identical to A1)
was cloned into a destination vector together with a miR173 target site, which can
trigger the production of trans-acting small interfering RNAs (tasiRNAs), so that the
expression of all three endogenous PP2A-A genes can be suppressed simultaneoulsy. Indeed, among the RNAi progenies, three independent transgenic
RNAi-a1;a2;a3 lines with reduced expression level of PP2A-A genes were isolated
(Fig. 5G). T3 plants showed abnormal pavement cell shape (Fig. 1H and Fig. 5E, F)
and reduced SI (RNAi-a1;a2;a3 #2=16.4%, n=2655; #4=16.1%, n=3117; #10=15.2%, n=3334 vs. WT = 23.7%, n=1628) (Fig. 1J). Phenotypic quantification showed that the stomatal defects of the RNAi plants were weaker than those of crispr-a1;a2;a3 #7 and #12 lines, but comparable to the double mutants a1;a2 and a1;a3 (Fig. 1J). Taken together, we collected multiple lines of genetic evidence that demonstrate a positive role of the PP2A phosphatases in stomatal production.

**PP2A Functions to Promote Stomatal Production in Arabidopsis**

As the PP2A protein phosphatases are heterotrimeric, disruptions of the catalytic PP2A-C subunits were anticipated to lead to similar developmental defects as those caused by the A mutations. Indeed, the c3;c4 double mutants, as previously reported, show a dwarfed growth phenotype, mirroring that of a1;a2 and a1;a3 mutants (23) as well as defects in pavement cell morphogenesis (26). We also found dramatically reduced stomatal density (Fig. 1I and J) in the leaf epidermis of the c3;c4 mutants, further demonstrating the positive role of PP2A in stomatal production.

Besides genetic strategies, we deployed pharmacological treatment to define PP2A’s function in stomatal development. Cantharidin (CT) is a widely-used PP2A inhibitor that preferentially inhibits protein phosphatases PP2A and PP1 (30). Wild-type seeds expressing the cell outline marker Q8 (GFP-tagged plasma membrane channel PIP2A) (31) were germinated on half-strength MS medium supplemented with 10 µM or 50 µM CT, while the corresponding amount of DMSO solvent was used as control. In 3 days-post-germination (3-dpg) seedlings, we found that CT treatment suppressed stomatal production (SI reduced to 17.3% by 10 µM CT n=1752, and to 2.7% by 50
µM CT n=1103, compared to 25.0% in the DMSO control experiments, n=1996) (Fig. 2J, L). In addition, CT treatment induced dwarfed seedlings with limited root growth, phenotypes resembling those of the crispr-a1;a2;a3 lines (Fig. 5K) in which PP2A functions were severely depleted.

**PP2A Functions Downstream of MAPKs but Upstream of SPCH**

To study the genetic relationship of PP2A with the other key components in the regulation of stomatal development (depicted in Fig. 2A), we introduced crispr-a1;a2;a3 mutations in a number of stomatal mutants. ERECTA (ER) is a receptor-like protein kinase, and the loss-of-function mutant er105 produces excessive asymmetric cell divisions and meristemoids (6). After the crispr-a1;a2;a3 constructs were introduced into the er105 mutant, the SI in er105 (15.0%, n=1362) was greatly reduced to 2.4% (n=1254) (Fig. 2B, C, and K), suggesting crispr-a1;a2;a3 is epistatic to er105 in stomatal production. The success of generating crispr-a1;a2;a3 mutations in these transgenic plants was manifested by a number of phenotypic features, including small and dark green leaves, short roots, and abnormal pavement cell shapes, etc.

Downstream of the cell-surface ER receptors, the MAPKKK YODA (YDA) functions as a molecular switch of stomatal identity in the epidermis (11). The null yda-3 mutation leads to excessive guard cell formation and severe stomata clustering (11). After the crispr-a1;a2;a3 construct was introduced into yda-3 mutant, the stomatal overproduction and clustering phenotype was greatly suppressed in the progeny, with SI reduced to 16.0% (n=3858) compared with 42.3% (n=1287) in yda-3 (Fig. 2D, E
and K), suggesting that crispr-a1; a2; a3 is epistatic and PP2A functions downstream of YDA.

MAPK3 and 6 redundantly function downstream of the MAPKKK YDA in stomatal development (12). We examined a chemically inducible loss-of-function double mutant of mpk3; mpk6, named as MPK6SR (32), which harbors the double loss-of-function T-DNA insertional mutations that are functionally complemented by a MPK6 variant (MPK6[YG]). In the presence of the chemical inhibitor NA-PP1 (4-amino-1-tert-butyl-3-(1′-naphthyl) pyrazolo [3,4-d] pyrimidine), the complementation of MPK6[YG] can be disrupted, resulting in the loss-of-function, severely clustered stomatal phenotype (12, 32). When crispr-a1; a2; a3 was introduced into MPK6SR, the NA-PP1 induction of stomatal overproliferation was significantly impaired (Fig. 2F, G, SI of 61.1% n=546 reduced to 7.3%, n=1553, quantification in Fig. 2K). These results indicate that crispr-a1; a2; a3 stomatal phenotype is epistatic to mpk3; 6. The above genetic results were further confirmed by the CT treatment of two dominant negative mutants of the pathway, DNyda (11) and DNmpk6 (33). In both cases, the severe stomatal clustering phenotype was alleviated by CT treatment (Fig. 7A, B), consistent with PP2A acting downstream of the YDA MAPK cascade.

In the nucleus, the bHLH transcriptional factor SPCH is the master regulator that initiates the stomatal lineage in Arabidopsis (1) and is directly targeted by the YDA MAPK cascade for degradation (13). The null spch-3 mutation eliminates stomatal production in the epidermis (1). When we introduced crispr-a1; a2; a3 mutations into spch-3, the progeny produced the stomata-less epidermis of spch-3 with round
pavement cells, a typical feature of crispr-a1;a2;a3 (Fig. 2H, I), suggesting that PP2A is not epistatic to SPCH and that PP2A functions somewhere between MAPKs and SPCH.

Inhibition of PP2A Suppresses Protein Expression of Key Transcription Factors

Stomatal development in Arabidopsis involves sequential events of 1) lineage initiation controlled by SPCH with partners SCRM/ICE1 and SCRM2 (4), 2) stomatal fate transition mediated by MUTE (2) and 3) terminal fate differentiation switched by FAMA (3). To determine which step in stomatal development is regulated by PP2A, we took advantage of the PP2A inhibitor CT to examine the responses of the corresponding marker lines. The Arabidopsis marker lines expressing SPCH-CFP (5), MUTE-GFP (2) and GFP-FAMA (3), all driven by their endogenous promoters, were germinated and grown in the presence of 50 µM CT, and the adaxial side of 3-dpg cotyledon epidermis were examined for protein accumulation. Because all these markers are cell type-specific and expressed transiently, the detection of such low abundance proteins in the whole-plant tissue by biochemical strategies, e.g. immunoblotting, has not been trivial. We therefore relied on counting the frequency of marker-expressing cells to indicate protein expression levels. We observed that the long-duration CT treatment (plants grown for 3 days) greatly reduced the percentage of cells expressing each of the three transcription factors, e.g. SPCH-CFP (dropped down to 3.0%, n=2048 CFP-positive cells in the CT treatment, compared to 18.5%, n=8112 in the DMSO control), MUTE-GFP (0.0% in CT, n=10776 vs. 9.0% in DMSO,
n=6080), and GFP-FAMA (1.8% in CT, n=1512 vs. 20.5% in DMSO, n=774) (Fig. 3A, B, C, quantification in Fig. 6A). Interestingly, the expression of the SPCH partner, SCRM/ICE1-YFP was not significantly affected by CT (Fig. 3D, quantification in Fig. 6A). To further test whether the loss of marker expression occurs at the transcription level, WT seedlings were germinated and grown with 50 µM CT or DMSO, and total RNAs from 3-dpg seedlings were extracted. Quantitative real-time PCR showed that the transcript levels of \textit{SPCH}, \textit{MUTE}, and \textit{FAMA} were significantly lowered by the CT treatment, while SCRM/ICE1 transcript level was not significantly changed (Fig. 6B). Thus, our results suggested that the CT-lowered expression of \textit{SPCH}, \textit{MUTE}, and \textit{FAMA} could occur at the transcriptional level.

Possible functional connections between PP2A and other stomatal factors were also examined in protein fusion lines, including the receptor-like protein TMM-GFP (7) (driven by the endogenous promoter), the MAPKKK YDA (YDA-YFP driven by its native promoter and the dominant-negative DNyda-YFP, equivalent to the kinase inactive version, driven by the 35S promoter), MPK6 (the kinase inactive DNmmpk6-YFP driven by the 35S promoter), the polarity factor BASL (GFP-BASL driven by its own promoter), and SCRM/ICE1-YFP (driven by its own promoter). We found that none of these markers were discernably changed by impaired PP2A function (Fig. 7A-F). These data are also consistent with our genetic analysis showing that PP2A functions downstream of the plasma membrane receptors and the MAPK cascade (described above).

\textbf{PP2A Promotes SPCH Protein Stability}
Because the early stomatal gene *SPCH* appeared to directly regulate key factors in cell division and cell fate determination and because chromatin immunoprecipitation assays supported the physical binding of SPCH with the promoter of *MUTE*, but not that of *FAMA* (5, 34), we suspected that the lowered expression of these genes in response to CT could result from lowered levels of SPCH protein. To test this possibility, we shortened the CT treatment duration to examine the immediate consequences on protein accumulation. The three translational marker lines (SPCH-CFP, MUTE-GFP and GFP-FAMA) were grown for 3 days, followed by short-term CT treatment (50 µM for 1-hr, 4-hr, and 24-hr, respectively). We found that SPCH-CFP was very sensitive to CT: after 1-hr of incubation, the percentage of cells expressing SPCH-CFP was reduced by 30% (13.8% in CT, n=2016 vs. 19.7%, n=884 in DMSO); after 4-hr, 70% of SPCH-CFP-positive cells were lost (5.2%, n=1670 vs. 19.7% in DMSO), and after 24-hr, SPCH-CFP were almost absent (only 1.1% CFP-positive, n=2865). In the DMSO control experiment, the number of cells expressing SPCH-CFP was maintained stably at all three time points (Fig. 8A, quantification in Fig. 8B). Interestingly, MUTE-GFP showed similar, though milder, responses to 50 µM CT: after 1-hr of incubation, the percentage of cells expressing MUTE-GFP was reduced by 21% (from 6.0% n=965 dropped to 4.7%, n=2309), after 4-hr, 46% of MUTE-GFP-positive cells were lost (3.2%, n=2888), and after 24-hr, 76% of MUTE-GFP were lost (1.3%, n=2249). Again, in the control experiment, MUTE-GFP expression remained stable in 24-hr DMSO (Fig. 9A, quantification in Fig. 9B). Strikingly, however, the expression levels of GFP-FAMA remained almost
unaffected at all the time points within 24-hr of 50 µM CT treatment (Fig. 9D, quantification in Fig. 9E). These results clearly demonstrated that the protein accumulation of SPCH and MUTE are sensitive to short-term CT-triggered protein phosphoregulation, but FAMA did not appear to be regulated by this mechanism. As both GFP- and CFP-tagged proteins were assayed in our experiments, we evaluated possible influences of different fluorescent protein (FP) tags to protein stability by testing the responsiveness of GFP-tagged SPCH (1) versus the CFP-tagged SPCH under the same treatment. Quantification data showed that SPCH-GFP responded similarly to SPCH-CFP in CT treatment with regards to their protein instability (Fig. 8E). Thus, different FP tags (GFP/YFP/CFP) were interchangeably used in our study.

We further evaluated whether the CT-triggered low expression of SPCH-CFP and MUTE-GFP occur at the transcription level by evaluating the responses of the transcriptional reporter lines (SPCHpro::nucYFP and MUTEpro::nucYFP). The data showed that, unlike the protein fusion, the expression of SPCHpro::nucYFP was not sensitive to CT and remained at equivalently high levels after 1, 4, and 24 hours of treatment (Fig. 8A, quantification in Fig. 8C). Furthermore, the protein fusion SPCH-CFP, but not the transcriptional fusion SPCHpro::nucYFP, was greatly reduced by crispr-a1;a2;a3 mutations and in a1;a3 mutants (Fig. 3E, F, I). This was also supported by quantitative PCR results demonstrating comparable transcript levels of SPCH-CFP in both WT and crispr-a1;a2;a3 backgrounds (Fig. 8D). Thus, our results suggest that the positive role of PP2A in the regulation of SPCH expression occurs at the protein level and not at the transcription level. In contrast to the stable expression
of SPCHpro::nucYFP, the expression of MUTEpro::nucYFP was very sensitive to CT treatment, with the reduction rates of 38% (from 17.1%, n=995 to 10.3%, n=1923) at 4-hr and 73% (from 17.1%, n=995 to 4.65%, n=2129) at 24-hr 50 µM CT treatment (Fig. 9A, quantification in Fig. 9C). In the same set of experiments, the DMSO controls showed much milder reductions (Fig. 9C). On the other hand, the ubiquitously expressed stomatal regulator SCR M/ICE1 was not affected by the CT-caused reduction of SPCH protein (Fig. 6B). Thus, the results indicated the specific impacts of the PP2A inhibitor CT on the expression of different stomatal genes: CT inhibits the protein accumulation of SPCH, while its suppression of MUTE most likely occurs at the transcriptional level, which may result from lowered SPCH abundance.

As SPCH was postulated to be phosphorylated and then degraded by the 26S proteasome system (14), we tested the hypothesis that PP2A might be the phosphatase counteracting this process. We combined the 26S proteasome inhibitor MG132 with CT in the treatments, anticipating that MG132 would abolish the CT-triggered protein instability of SPCH. Indeed, our data showed that 24-hr 50 µM MG132 treatment almost fully recovered robust SPCH-CFP expression in CT-treated 3-dpg seedlings (Fig. 3G, H). Thus we propose that PP2A, likely through protein dephosphorylation, promotes the stability of SPCH protein which in turn promotes MUTE transcription and stomatal production in Arabidopsis.

**PP2A Regulates the Phosphorylation Status of SPCH**

The SPCH sequence contains numerous possible phosphorylation sites for MPK3/6, CDKA;1 and BIN2 (13-15); many of these sites are distributed in the unique MAPK
targeting domain (MPKTD), with a few additional ones in the N-terminal domain (Fig. 9F). It was reported that overexpression of the SPCH variant with the MPKTD deleted (SPCHΔ93) generated large clusters of stomata (13), presumably due to low phosphorylation levels, thus elevated protein stability and activity of SPCH. We further mutated the putative N-terminal Ser/Thr phosphorylation sites to Alanines to produce an enhanced phospho-deficient version, SPCHΔ93Si/T38-44A-S65A. If PP2A indeed functions to lower SPCH phosphorylation level, the phospho-deficient SPCHΔ93Si/T38-44A-S65A should be less sensitive to PP2A activity in plants. To test this hypothesis, we introduced SPCHΔ93Si/T38-44A-S65A-YFP driven by the SPCH promoter into plants and found that, compared to the WT SPCH-CFP (which only weakly stimulates stomatal overproduction; (13), the expression of SPCHΔ93Si/T38-44A-S65A-YFP led to severe over-proliferation of stomata and responded to CT less sensitively. The quantification data showed that, compared to the nearly 100% reduction of SPCH-CFP expression by 24-hr CT treatment (n=2865; Fig. 3I, J), CT-treated SPCHΔ93Si/T38-44A-S65A-YFP plants showed only a 20% reduction (n=1915) (Fig. 3I-J). Similarly, while the SPCH-CFP expression was greatly reduced in a1;a3 mutants, SPCHΔ93Si/T38-44A-S65A-YFP maintained high expression levels (Fig. 3I). These results collectively demonstrate that the SPCH protein level is very sensitive to PP2A activity, and suggest that PP2A may regulate SPCH phosphorylation status.

The PP2A-A Subunits Physically Interact with SPCH

Supported by the above experimental evidence, we asked if PP2A directly targets the SPCH protein to regulate its phosphorylation status. Canonical PP2A complexes are
composed of one A (scaffolding), one B (regulatory), and one C (catalytic) subunit, and in Arabidopsis each subunit type is encoded by multiple genes (Fig. 1A) (16). A non-canonical complex containing a C subunit bound by the TAP42/alpha4/TAP46 regulatory protein has also been identified in plants and other eukaryotes (35-37). To determine which subunit[s] may directly interact with SPCH, based on their expression profiles (38) and sequence similarity analysis, we selected PP2A subunits (A1, A2, A3, B^β/B4, B^α/B13, FASS/TON2/B12, C1 and TAP46) and tested their ability to interact with SPCH in planta, in yeast, and in vitro. Bimolecular fluorescence complementation (BiFC) assays indicated that SPCH associates with A1, A2 and A3 in tobacco epidermal cells (Fig. 4A-F, Fig. 10F-G). It is worth noting that, although the PP2A-A subunits were ubiquitously distributed/localized at the subcellular level in tobacco epidermal cells (Fig. 10B-D), their interaction with SPCH occurred only in the nucleus (Fig. 4A-F, Fig. 10E-G). Similarly, the yeast two-hybrid assay detected the interaction of SPCH with A1 and A2 (Fig. 4G), but not with the B and C subunits (Fig. 10A). The physical association between SPCH and A1/A2 was further confirmed by pull-down assays with recombinant proteins in vitro (Fig. 4H). Taken together, these results strongly support our hypothesis that PP2A-A subunits may directly bind to SPCH, allowing dephosphorylation of SPCH, and thus combating the activities of protein kinases, e.g. MAPKs and BIN2 (Fig. 4I).

Discussion

Stomatal production is not only vital for plant growth and development, it is also highly
related to the ecosystem water balance on the earth. Strong genetic evidence supports the crucial role of SPCH in the initiation of the stomatal lineage cells in both monocots (39, 40) and dicots (1, 2). Stomatal production is highly plastic in responding to environmental changes that necessitate SPCH expression levels to be highly adjustable. Therefore, it is not surprising that a number of studies demonstrated that the regulation of SPCH expression and function occurs at multiple levels. For example, the SPCH promoter is directly targeted and transcription is suppressed by the bHLH transcription factor PIF4, a core regulator in high-temperature signaling (41, 42). The C2H2 zinc finger IDD16 transcription factor that functions in plant organ morphogenesis also directly suppresses SPCH expression (43). At the protein level, SPCH stability and function both are tightly related to its phosphorylation status. In Arabidopsis, both MPK3/6 and the GSK3-like kinase BIN2 target SPCH for phosphorylation and degradation (Fig. 9F) (13, 14). On the other hand, the phosphorylation of SPCH can also be positive for its function. For example, the CDKA;1 kinase phosphorylates SPCH on one specific amino acid that seemed to promote SPCH’s activity in stomatal divisions (15). Here, we found that PP2A stabilizes SPCH and promote its accumulation, counteracting kinase-mediated SPCH phosphorylation and the resulting protein degradation. It remains unknown whether PP2A-mediated SPCH dephosphorylation may alter functional specificity and/or activity of this bHLH factor in transcription. A previous study showed that PP2A dephosphorylates the bHLH factor HAND1 to regulate its dimerization and functional specificity in limb development (44). In stomatal development, the bHLH SCRM/ICE1
scaffolds the physical interaction between MAPKs and SPCH (45) and SCRM protein stability is also regulated by protein phosphorylation (46). Although our research did not suggest that PP2A dephosphorylates SCRM/ICE1, future research should address the question of whether heterodimerization of SPCH and SCRM/ICE1 is regulated by PP2A-mediated SPCH dephosphorylation.

Posttranslational modifications, including protein phosphorylation, are dynamically modulated by opposing regulators, such as kinases and phosphatases. With several kinases identified to phosphorylate SPCH, how SPCH dephosphorylation is regulated has been unknown before our study. While the Arabidopsis genome encodes more than 150 protein phosphatase catalytic subunit genes (47-49), PP2A family phosphatases constitute a major group of protein Ser/Thr phosphatases and are implicated in many biological processes (18). From our study, it is intriguing to note that PP2A appeared to be functionally coupled with a few highly conserved kinases, such as MAPKs and the GSK3-like kinases, to balance protein phosphorylation status in multiple pathways. For example, the BR-responsive transcription factor BZR1 is phosphorylated and inactivated by BIN2 in the absence of BR, but in the presence of BR, BZR1 is activated by PP2A-mediated dephosphorylation, which relieves the BIN2-mediated inhibition of BR responses (50, 51). The key ethylene biosynthetic enzymes, 1-aminocyclopropane 1-carboxylate synthases 2 and 6 (ACS2/6), are phosphorylated (and stabilized) by MPK6 (52, 53) and dephosphorylated (and destabilized) by PP2A (54). In mammals, the p70 ribosomal protein S6 kinase (p70S6K) regulates cell growth, proliferation and cell differentiation, and its
phosphorylation status is tightly controlled by the antagonistic actions of the GSK3β kinase and PP2A (55). Here, our study discovered the opposing functions of PP2A with MAPKs and GSK3-like kinases, providing another compelling example of kinase-phosphatase antagonism in the control of stomatal development, and underscoring the sophisticated signaling network that shapes this highly plastic plant developmental program.

Stomatal development and functional behavior are highly responsive to environmental cues (56). Genetic evidence showed that light, temperature, and carbon dioxide are influential to stomatal production and the corresponding key regulators have been identified (46, 57-59). PP2A function has been tightly linked to plant responses to environmental changes/stresses and developmental cues (20, 25, 27, 51). Our studies have placed PP2A downstream of the MAPK cascade but upstream of SPCH, likely by direct phosphoregulation of SPCH. Therefore, it is tempting to speculate that PP2A transduces environmental and/or developmental cues to optimize the SPCH protein level, thus stomatal production. In the past, phosphatases were often considered as housekeeping dephosphorylation enzymes, without much specificity, however, they are now known to be both highly specific and subject to sophisticated regulatory mechanisms (18, 60). In our study, we detected direct physical binding between SPCH and the scaffolding A subunit of PP2A, differing from many PP2A-substrate interactions that are largely controlled by the B regulatory subunits (61). Interestingly, it was recently found that small Rab GTPases, prominent regulators of vesicular trafficking, interact with the A subunits and may compete with
the catalytic subunit for the formation of a functional PP2A complex (62). Whether SPCH may, by binding to the A subunits, regulate PP2A’s function in the nucleus will be an intriguing research direction in the future. On another hand, the microarray dataset indicated that several genes encoding PP2A subunits, particularly the B subunits and some C subunits, are responsive to environmental factors, e.g. abiotic and biotic stresses, chemical treatments and light (19). Future study will focus on investigating under what conditions PP2A phosphatases are activated or inactivated, and how this regulation feeds into the PP2A-SPCH connection in stomatal development, and on defining how functional specificity is achieved by these convoluted signaling networks. A systematic investigation of expression pattern, subcellular localization, and genetic redundancy of the B and C subunits will be helpful to further characterize the functional specificity of PP2A in different developmental contexts.

Methods

Plant materials and growth conditions

*Arabidopsis thaliana* Col-0 was used as the wild-type (WT) unless otherwise noted. The mutant and marker lines used in this study were *er105* (CS89504), *yda-3* (Salk_105078), MPK6SR (32), spch-3 (1), DNyda (63), and DNmpk6 (63). The PP2A-A mutant lines a1 (rcn1-6, SALK_055903) (64), a1;a2 and a1;a3 lines were constructed by crossing rcn1-6 with the a2-1 (SALK_042724) and a3-1 (SALK_014113) mutants (24). The c3;c4 seeds (23) were the generous gift of Dr.
Martine Pastuglia.

Unless otherwise noted, Arabidopsis seeds were germinated on half-strength Murashige and Skoog (MS) medium at 22°C in constant light for 6-10 days. Seedlings were then transferred to potting mix for growth in 22°C growth chambers with 16-hr light/8-hr dark cycles.


**Cantharidin treatment**

The PP2A inhibitor cantharidin (CT, MilliporeSigma) was dissolved in DMSO for storage and usage. For long-duration CT treatment, Arabidopsis seeds were germinated and grown on half-strength MS/Agar medium supplemented with 10 µM or 50 µM CT, or with the corresponding amount of the DMSO solvent (mock). Seedlings were grown at 22°C in constant light for 3-5 days before images were captured. For short-term treatment, Arabidopsis seeds were grown on half-strength MS medium at 22°C in constant light for 3 days, followed by submerging into sterile water with the specified amount of CT and/or DMSO for treatment. Plant samples were kept in 22°C growth chambers for 1-hr, 4-hr, or 24-hr before images were captured.

**Plasmid construction and primer design**
The LR Clonase II-based gateway cloning technology (Invitrogen) was used to generate most constructs unless otherwise noted. The promoter fragments (approximately 2kb upstream of start codon), coding regions or genomic DNA of PP2AA genes were first cloned from Arabidopsis cDNA/gDNA (from Arabidopsis adult rosette leaves) to pENTR/D-TOPO vector (Invitrogen) and subsequently subcloned into binary destination vectors. Primers used to amplify these sequences are listed in Table S1. To introduce amino acid mutations into SPCH, the plasmid pENTR/D-TOPO carrying SPCH CDS without the stop codon was used as template and specific mutations were introduced by QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). The binary vector pMDC43 (65), with the 35S promoter replaced by an endogenous promoter, was used for PP2A-A overexpression. To generate the BiFC constructs for tobacco transient expression, pENTR/D-TOPO vectors containing the coding sequences of SPCH, PP2A-A1, PP2A-A2, PP2A-A3, PP2A-C1 SCRMI/ICE1 were recombined to destination vectors pH35YG, pH35GY (66), pXNGW, pNXGW, pXCGW, and/or pCXGW(67). The pENTR/D-TOPO carrying PP2A-A promoters were recombined to pBGYN backbone (68) to generate PP2A-Apro::nucYFP. Plasmids were transformed into Agrobacterium tumefaciens strain GV3101 for Arabidopsis transformation and tobacco leaf infiltration.

To generate RNAi-PP2A-A mutants, the protocol described in (29) was followed. Specifically, a 481-bp highly conserved region among the three PP2A-A genes was amplified from the A2 gene by RNAi-a1;a2;a3 primers and cloned into pCAMBIA3300 together with the miRNA173 target site by overlapping PCR. To generate
crispr-a1;a2;a3 mutants, we followed the protocol described in (28). Specifically, two pairs of oligos 100% matching PP2A-A1 and PP2A-A2/3, respectively, were designed. The sgRNA oligos were phosphorylated by T4 PNK (NEB) and annealed in thermocycler, followed by ligation with pMD18-T containing pAtU6-pAtUBQ-Cas9 cascade into the BbsI site. Then the chimeric AtU6-crispr-a1-pAtUBQ-Cas9 cassette was cloned into pCAMBIA1300 or pCAMBIA2300 through HindIII and EcoRI restriction sites to obtain p1300-crispr-a1 and p2300-crispr-a1, respectively. The AtU6-crispr-a2/3-pAtUBQ-Cas9 cascade was generated similarly except that the pAtU6-crispr-a2/3 cassette was inserted into p1300-crispr-a1 or p2300-crispr-a1 through KpnI and EcoRI sites to obtain p1300-crispr-a1;a2;a3 and p2300-crispr-a1;a2;a3, respectively. To genotype the crispr-a1;a2;a3 mutants, primers flanking the gRNA-targeting sites were designed to amplify the mutagenized regions of the three A genes. The amplicons were first assayed by gel electrophoresis. Single DNA bands were sent for Sanger sequencing. Heterogeneous amplicons were cloned in a TA cloning vector and individual clones (10-20 for each PCR reaction) were sent for Sanger sequencing. Primer sequences used in this study are listed in Table S1.

Confocal imaging and image processing

All confocal images were captured with a Leica TCS SP5 II confocal microscope. Unless otherwise noted, adaxial epidermis from cotyledons of 3-dpg (day-post-germination) Arabidopsis seedlings were used. Cells outlines were visualized by the propidium iodide (PI, Invitrogen) staining. Fluorescent proteins were
excited at 458 nm (CFP), 488 nm (GFP), 514 nm (YFP), 543 nm (mCherry) and 594 nm (PI). Emissions were collected at 480-500 nm (CFP), 501-528 nm (GFP), 520-540 nm (YFP), 600-620 nm (mCherry), and 591-636 nm (PI). Confocal images were false colored and brightness/contrast was adjusted with Fiji ImageJ software.

**Quantitative analysis of stomatal phenotype and SPCH protein level**

For quantification analysis of stomatal phenotype, 5-dpg seedlings were stained with propodium iodide (PI, Invitrogen) and adaxial cotyledon images were captured with a Carl Zeiss Axio Scope A1 fluorescence microscope equipped with a ProgRes camera (Jenoptik). Stomatal index (SI) was calculated as the percentage of stomatal guard cell number relative to the total number of epidermal cells. The student’s $t$-test was used to determine if difference is significant between lines.

To quantify SPCH protein expression levels, frequencies of CFP/YFP-positive cells were counted for comparison. Seedlings expressing SPCH-CFP and other variants were stained with PI and confocal images were captured from adaxial cotyledon epidermis. The number of CFP/YFP-positive cells and total epidermal cells were counted with Fiji ImageJ software, and the ratio of CFP/YFP-positive cells among total epidermal cells were calculated. The student’s $t$-test was used to determine if the difference is significant between treatments.

**RNA extraction and real-time PCR**

Total RNAs were extracted from 5-dpg Arabidopsis seedlings using RNeasy Plant Mini Kit (Qiagen). The first-strand cDNAs were synthesized by the qScript cDNA SuperMix (Quantabio) with 500 ng of total RNAs as template.
For real-time PCR assay, reactions were prepared by a SYBR Green Master Mix kit (ThermoFisher) and conducted by the StepOnePlus Real-Time PCR System (ThermoFisher). Three technical replicates were performed for each of three biological replicates. Expression values were normalized to the reference gene ACTIN2 using the ΔCT method. Data are presented as mean ± SD. Primers used in this study were listed in Table S1. The CFP primers used in this study to evaluate the expression level of SPCH-CFP were as described (69).

**Yeast two-hybrid assay**

The pENTR/D-TOPO vector containing coding sequences of SPCH, SCRM/ICE1, PP2A-A, PP2A-B and PP2A-C genes were cloned into bait vector pGBKT7 or prey vector pGADT7 (Clontech). The EZ-Transformation Kit (MP Bio-medicals) was used for yeast transformation by following the manufacturer’s instructions. The bait and prey plasmids were co-transformed into the yeast strain AH109 and positive transformants were selected with SD/-Leu/-Trp. The interactions were tested on SD/-Leu/-Trp/-His medium supplemented with appropriate concentration of 3-amino-1,2,4-triazole (3-AT).

**Transient expression in Nicotiana benthamiana and BiFC assay**

*Agrobacterium tumefaciens* strain GV3101 carrying binary constructs was cultured overnight in 10 mL Luria-Bertani (LB) medium with appropriate antibiotics. Agrobacterial cells were collected at 3,000 rpm for 10 min and resuspended in 10 mL of 10 mM MgCl2, followed by washing with 10 mM MgCl2, then OD600 of cell cultures were measured. For single construct transient expression assay, cell cultures were
diluted to final OD600 of 0.5. For the bimolecular fluorescence complementation (BiFC) assay, cell cultures of the BiFC pairs were equally mixed with p19, followed by final dilution to OD600 at 0.5. In both assays, mixed cell cultures were then infiltrated to 4-week-old tobacco leaves. Leaf disks were excited 2-3 days after infiltration and fluorescence was checked with the confocal microscope. Three independent experiments were performed to obtain the representative images.

**Recombinant protein production and pull-down assay**

*E. coli* expression, protein induction and purification of His-PP2A-A1, His-PP2A-A2, MBP, and MBP-SPCH were performed using standard protocols. For the pull-down assays, 10 µg of His-PP2A-A1/PP2A-A2 proteins were incubated with pre-washed MBP or MBP-SPCH beads in 1 mL of TBST buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) overnight at 4°C with gentle shaking. Then, the beads were collected and washed with the TBST buffer for 3-5 times to eliminate nonspecific bindings. The beads were then boiled and loaded on a SDS-PAGE gel, and the pull-down proteins were analyzed by Western Blot immunoblotting with the α-His antibody (Cell Signaling Technology), and input proteins were analyzed by the α-MBP antibody (NEB). The pull-down assays were repeated at least 3 times to acquire the representative image.

**Acknowledgement**

We thank our lab members for critical readings of the manuscript. We appreciate Dr. Martine Pastuglia (French National Institute of Agricultural Research) and Dr. Haiyang
Wang (Chinese Academy of Agricultural Sciences) for contributing genetic materials.

We thank the Frommer Lab (previously at Carnegie, Stanford) for sharing the BiFC constructs. This work is supported by grants from the National Institute of Health (R01GM109080) to J.D. C.B. is supported by the China Scholarship Council and Rutgers University.
Fig. 1. PP2A promotes stomatal production in Arabidopsis

(A) Diagram depicting a heterotrimeric PP2A complex. Numbers in parentheses indicate the number of genes in the Arabidopsis genome encoding the corresponding subunit type. (B-D) DIC images of 5-dpg cotyledons in Col (B), a1;a2 (C), and a1;a3 (D) seedlings. (E-I) Confocal images of 3-dpg adaxial epidermis of cotyledons in Col (E); crucra-a1;a2;a3 #7 (F) and #12 (G); RNAi-a1;a2;a3 #2 (H); and c3;c4 (I). (J) Quantification of stomatal index (SI) in 5-dpg adaxial cotyledon epidermis. Data are mean ± SD. n=930-3334 total epidermal cells of 10-12 individual plants. *significantly different compared with the WT (Col) values (Student’s t-test, *P < 0.05, **P < 0.001). n.s: not significant. Guard cells are manually traced with green highlights to improve visibility. Cell walls were stained with propidium iodide (PI) in red. Scale bars represent 50 µm.
Fig. 2. PP2A functions downstream of MAPKs but upstream of SPCH

(A) Simplified diagram to describe key signaling molecules in stomatal development. ERF, the ERECTA receptor-like kinase family; YDA, the MAPKK Kinase; MPK3/6, MAPK 3 and 6; SPCH, the bHLH transcription factor. PM, plasma membrane; NE, nuclear envelope. (B-G) Confocal images of 3-dpg seedlings of er105 (B), er105;crispr-a1;a2;a3 (C), yda (D), yda;crispr-a1;a2;a3 (E), mpk3;6 (F), and mpk3;6;crispr-a1;a2;a3 (G). (H-I) DIC images of 5-dpg seedlings of spch-3 (H) and spch-3;crispr-a1;a2;a3 (I). (J) Confocal images of 3-dpg Q8 seedlings (GFP expression in green) grown with DMSO, 10 µM and 50 µM CT. (K) Quantification of SI in 3-dpg seedlings shown in (B-G). (L) Quantification of SI in 3-dpg adaxial cotyledons shown in (J). Data are mean ± SD. n=546-3858 cells in (K), and 1103-1996 cells in (L). *significantly different from the WT values (Student’s t-test, *P < 0.05, **P < 0.001). Cell outlines were visualized by PI staining (red). Scale bars in (B-J) represent 50 µm.
Fig. 3. PP2A promotes SPCH protein stability

(A-D) Confocal images of 3-dpg seedlings expressing SPCH-CFP (A), MUTE-GFP (B), GFP-FAMA (C), and SCRM/ICE1-YFP (D) grown with DMSO and 50 µM PP2A inhibitor CT. (E-F) 3-dpg cotyledons of the translational fusion SPCHpro::SPCH-CFP (E) vs. the transcriptional fusion SPCHpro::nucYFP (F) seedlings in the WT background (left panels) and crispr-a1;a2;a3 background (right panels). (G) Confocal images of 3-dpg SPCH-CFP seedlings incubated 24-hr with DMSO, 50 µM CT, and 50 µM CT mixed with 50 µM MG132, respectively. (H) Quantification of CFP-positive cells in total epidermal cells shown in (G), n=798-2865 cells from 10-12 seedlings. (I) Confocal images of 3-dpg SPCH-CFP (upper) and SPCHΔ933/S38-44A-S65A-YFP (lower) seedlings incubated 24-hr with DMSO (left), 50 µM CT (middle), and in a1;a3 (right). (J) Quantification of the adjusted ratios of fluorescence-positive cells in SPCH-CFP.
and SPCHΔS/T38-44A-S65A-YFP in 3-dpg cotyledons in responding to DMSO and 50 µM CT at different time point (0-, 1-, 4-, and 24-hr after treatment), n=1915-2865 cells. The initial ratios of FP-positive cells were defined as 100%, and the relative ratios (adjusted) were calculated at each timepoint. In the confocal images, the expression GFP/YFP/CFP were artificially colored with green and cell outlines were visualized by PI staining (red). Scale bars represent 50 µm. Data are mean ± SD. *significantly different from the WT control values (Student's t-test, *P < 0.05, **P < 0.001). n.s: not significant.
Fig. 4. PP2A-A subunits directly bind to SPCH

(A-F) Confocal images of BiFC assays to test for interactions between SPCH and the PP2A-A subunits. The expression of half YFPs (YFP^N and YFP^C) were used as negative controls. SCRM/ICE1 was used as positive control. Scale bars represent 50 µm. (G) Yeast two-hybrid assay for BD-SPCH with PP2A-A (AD-A1, and A2). The BD and AD empty vectors were used as negative control and SCRM/ICE1 was used as positive control. Yeast growth controls are shown on the left side of each pair of panels (-Leu-Trp). Interaction tests are shown on the right side (-Leu-Trp-His with 3-AT). (H) In vitro pull-down assays using recombinant proteins, MBP-SPCH and His-PP2A-A subunits, with MBP-only being used as negative control. Immunoblots were visualized by anti-His. MBP-tagged proteins were stained by Coomassie brilliant blue (CBB). (I) Diagram shows a working model for PP2A in the regulation of SPCH to promote stomatal development. PP2A might function in opposition to the identified kinases (MAPKs and BIN2) to balance the phosphorylation status of SPCH in the initiation of stomatal lineage cells.
Fig. 5. Stomatal phenotypes and genetic characterization of pp2a-a mutants
(A) Gene structure of three PP2A-As with exons (black), introns (thin lines) and UTRs (gray). Sites targeted by sgRNA1 and sgRNA2 are indicated by red and blue triangles, respectively. RNAi targeting sites are indicated by purple lines. (B-D) DIC images of 5-dpg adaxial side cotyledons of Col (B), a1 (C), and a2;a3 (D) seedlings. (E-F) Confocal images of 3-dpg cotyledon epidermis in RNAi-a1;a2;a3 #4 (E) and #10 (F). Guard cells are manually traced with green highlights in (E-F) to improve visibility. (G) Quantitative real-time PCR analysis for transcript levels of A1, A2 and A3 in 5-dpg
seedlings. ACTIN2 was used as an internal reference. Three biological replicates were performed. Data are mean ± SD. *significantly different compared to the WT (Col) values (Student's t-test, *P < 0.05, **P < 0.001). (H-J) Confocal images of 3-dpg adaxial epidermis of cotyledons expressing A1pro::mCherry-A1 (H), A2pro::mCherry-A2 (I), and A3pro::mCherry-A3 (J). Cell outlines are visualized by PI staining (red) in (E-F and H-J). (K) Comparison of adult plant morphology of Col, T1 individuals of crispr-a1;a2;a3 #7 and #12. (L) Mutations detected (and the resulting predicted amino acid changes) in T2 plants of crispr-a1;a2;a3 #7 and #12. Genotype data for crispr-a1;a2;a3 plants were obtained as described in the Methods. Scale bars represent 50 µm.
Fig. 6. Quantification of bHLH expression in response to CT treatment.
(A) Quantification of fluorescence-positive cells in 3-dpg cotyledons of SPCH-CFP, MUTE-GFP, GFP-FAMA, and SCRM/ICE1-YFP grown on 1/2 MS medium with DMSO or 50 µM CT. \( n=774-8112 \) cells from 10-12 individual plants. (B) Quantitative real-time PCR assay to detect transcript levels of the designated genes in 3-dpg WT seedlings. ACTIN2 was used as an internal reference. Three biological replicates were performed. Data are mean ± SD. *significantly different from the DMSO control values (Student’s \( t \)-test, \( *P < 0.05, **P < 0.001 \)). n.s: non-significant.
Fig. 7. Expression patterns of stomatal regulators when PP2A activities are defective.

(A-F) Confocal images of 3-dpg cotyledons in 35S::DNyda-YFP (A) and 35S::DNmpk6-YFP (B) seedlings grown on 1/2 MS medium with DMSO (left panels) or 50 µM CT (right panels). (C-F) Confocal images of 3-dpg seedlings expressing SCRM/ICE1-YFP (C), YDA-YFP (D), TMM-GFP (E) and GFP-BASL (F) transgenes in the Col (left panels) and crispr-a1;a2;a3 backgrounds (right panels). Cell outlines are visualized by propidium iodide (PI) staining (red) and GFP/YFP are shown in green. Scale bars represent 50 µm.
Fig. 8. SPCH stability is reduced by defective PP2A function

(A) Confocal images of 3-dpg adaxial side cotyledons of SPCH-CFP and SPCHpro::nucYFP (green) treated with DMSO or 50 µM CT for 1h, 4h, and 24h, respectively. Cell outlines are visualized by PI staining (red). (B-C) Quantification of CFP- or YFP-positive cells in 3-dpg SPCHpro::SPCH-CFP, n=884-2865 cells (B) and SPCHpro::nucYFP, n=650-1482 cells (C). Seedlings were treated with the DMSO control or 50 µM CT for 0h, 1h, 4h, and 24h, respectively. (D) Quantitative real-time PCR assay for transcript levels of SPCH-CFP in the WT and crispr-a1;a2;a3, respectively. ACTIN2 was used as an internal reference. Three biological replicates were performed. (E) Quantification of GFP/CFP-positive cells in SPCHpro::SPCH-CFP and SPCHpro::SPCH-GFP 3-dpg cotyledons. Seedlings were treated with DMSO or 50 µM CT. Confocal images were captured at 0-, 1-, 4-, and 24-hr after treatment. n=1247-3571 cells in 10-12 individual seedlings. The Adjusted Ratios were generated by defining the initial percentages of CFP/GFP-positive cells as 100%, then the relative ratios at each time point were calculated accordingly. Scale
bars represent 50 µm. Data are mean ± SD. *significantly different from the DMSO control values (B-C) and from WT (Col) value (D) (Student's t-test, *P < 0.05, **P < 0.001). n.s: non-significant.
Fig. 9. Differential responses of key bHLH transcription factors to defective PP2A function

(A) Confocal images of 3-dpg adaxial cotyledons expressing the translational fusion MUTE-GFP and the transcriptional fusion MUTEpro::nucYFP. Cell outlines are

(B) Analysis of GFP-positive cells over time for MUTE-GFP with DMSO and 50uM CT.

(C) Analysis of GFP-positive cells over time for MUTEpro::nucYFP with DMSO and 50uM CT.

(D) Confocal images of GFP-FAMA with DMSO and 50uM CT.

(E) Analysis of GFP-positive cells over time for GFP-FAMA with DMSO and 50uM CT.

(F) Diagram of SPCH35/734-46A-655A with sites for MAPK-targeting.
visualized by PI staining (red) and GFP expression are in green. Seedlings were treated with DMSO or 50 µM CT for 1-hr, 4-hr, and 24-hr, respectively. (B-C) Quantification of GFP/YFP-positive cells at different time point in 3-dpg MUTE-GFP, n=965-2888 cells (B) and MUTEpro::nucYFP, n=699-2129 cells counted for each time point (C). Seedlings were treated with DMSO or 50 µM CT for 0-, 1-, 4-, and 24-hr, respectively. (D) Confocal images of 3-dpg seedlings expressing the translational fusion GFP-FAMA in responding to DMSO or 50 µM CT at 1-hr, 4-hr, and 24-hr, respectively. (E) Quantification of GFP-positive cells in 3-dpg seedlings expressing GFP-FAMA in responding to DMSO or 50 µM CT for 0-hr, 1-hr, 4-hr, and 24-hr. n=794-2256 cells counted for each time point. Quantification data (B, C, and E) are mean ± SD. *significantly different from the DMSO control values (Student's t-test, *P < 0.05, **P < 0.001). n.s: not significant. Scale bars in (A) and (D) represent 50 µm. (F) Diagram for SPCH subdomain with identified phosphorylation sites (vertical lines, color coded as indicated). SPCHΔ93S/T38-44A-S65A is a variant with the MAPK-targeting domain deleted (Δ93) and a few N-terminal phosphorylation sites (S/T, S38, T40, T43, and S65) mutated to Ala to make S/T38-44A-S65A.
Fig. 10. Interaction assays for SPCH with the PP2A subunits

(A) Yeast two-hybrid assay to test BD-SPCH interaction with AD-B13α, AD-B13β, AD-B13B', AD-B13TAP46, AD-B13TON2, AD-B13ICE1, and AD-B13C1. BD and AD empty vectors were used as negative controls, SCRM/ICE1, a known binding partner of SPCH, was used as positive control. Left, growth controls (-Leu-Trp). Right, interaction tests (-Leu-Trp-His with 2.5 mm 3-AT).

(B-E) Confocal images to show transient protein expression patterns of YFP-A1 (B), YFP-A2 (C), YFP-A3 (D), and CFP-SPCH (E) in tobacco epidermal cells. (F-G) Confocal images of BiFC assays. Complemented YFP expression (yellow) was shown when SPCH-YFPN coexpressed with A3-YFPC (G). The YFPN co-expressed with A3-YFPC was used as a negative control (F). Scale bars represent 50 µm.
Table S1. Primers used in this study

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F-seq2-PDF1  CTT GAT GAT CCC ATT TAC TTC TCT  \textit{crispr mutant genotyping}

F-RCN1-NotI  GCTCC GCGGCCGC ATG GCT ATG GTA

R-Seq-PDF1  TCT GCA ACA TAC ACA ATC CAA CC

F-Seq-PDF2  GTG GCT GAT GTG TAA TAG GCA AG

R-Seq-PDF2  CCT AAA GAT GAC AGT GAC TAG GAC G  \textit{crispr mutant genotyping}

F-RCN1-NotI  GCTCC GCGGCCGC ATG GCT ATG GTA

GAT GAA CCG TTG

A1 coding sequence cloning

R-RCN1-Ascl  (NO STOP) A GGCGCGCC GGA TTG TGC TGC TGT

GGA AC

F-PDF1-NotI  GCTCC GCGGCCGC ATG TCT ATG ATC

GAT GAG CCG TTG

A2 coding sequence cloning

R-PDF1-Ascl  (NO STOP) A GGCGCGCC GCT AGA CAT CAT CAC

ATT GTC AAT AGA TTG

F-PDF2-NotI  GCTCC GCGGCCGC ATG TCT ATG GTT

GAT GAG CCT TTA TAC C

A3 coding sequence cloning

R-PDF2-Ascl  (NO STOP) A GGCGCGCC GCT AGA CAT CAT CAC

ATT GTC AAT AG

F-PP2A-C1-Notl  GCTCC GCGGCCGC ATG CCG TTA AAC

GGA GAT CTC G

PP2A-C1 coding sequence cloning

R-PP2A-C1-Ascl  A GGCGCGCC CAA AAA ATA ATC AGG

GGT CTT GCG C

cloning

F-TAP46-NotI  GCTCC GCGGCCGC ATG GGT GGT TTG

GCT ATG G

TAP46 coding sequence cloning

R-TAP46-Ascl (-STOP)  A GGCGCGCC GCC ACA AGG TGT GAG

TTT CTT G

F-B''α-Notl  GCTCC GCGGCCGC ATG GAA ATC GAT

B''α coding sequence cloning
GGT GGA AAC GAT G

R- B”α-Ascl (-STOP)  A GGC CGCC AAA TGG AGA TTC GAG
TGG TTC ATC C

F-TON2-NotI   GCTCC GCGGCCGC ATG TAT AGC GGA
TCT AGC GAT GGT G

R-TON2-Ascl (-STOP) A GGC CG GCC CTG AGA CTC TTC
AGG TGG T

F-B4-NotI  GCTCC GCGGCCGC ATG TTT AAG AAA
ATC ATG AAA GGT GGG C

R-B4-Ascl (no stop) A GGC CG GCC GGA AGT GAT CAT ATG
ATC TTC TTC TCC

F-SPCH-38-40-43-44A GGT GCCGGAGAGATAGCTCCGGCAGCT
GCAGCTGCACCTAAAGATGG
AACCACAAG

R-SPCH-38-40-43-44A CCATCTTTAGGTGCAGCTGCAGCTGCCG
GAGCTATCTCTCCGGCACCTTCAAGAC

F-SPCH-65A  GATCAAGATTATGAAAACTCAGCTCCTA
AGAGGAAAAAGCAAAG

R-SPCH-65A  CTT TGTTTTTCTCTTTAGGAGCTGAGTT
TTCATAATCTTGATCC

F-proPP2AA1 GTC TTG TTT TGT TTG TGC TTT CC
R-proPP2AA1 CTT ATG TGA AAG TTC GAA TCA AAT A1 promoter cloning
CAC

F-proPP2AA2 CGT ATT CAT AGT TCC TGA GAT G TGA G
R-proPP2AA2 CTT CAA CAA CAC CAA CAA CAA AAT A2 promoter cloning
TAC

F-proPP2AA3 GTT GTA CAG TTG CAT ATG TGT GTG A3 promoter cloning
R2-proPP2AA3 GTC GAT AAG CAC AGC AAT CGG
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CHAPTER THREE

The D6 protein kinases regulate stomatal development and patterning in Arabidopsis

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This chapter presents the collaborated work by C. Bian and K. Yang.
Abstract

BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) is a plant-specific protein that is uniquely polarized to the cell cortex of the stomatal lineage cells in Arabidopsis (1). A basl loss-of-function mutant generates abnormal stomata clusters and bears asymmetric cell division defects (1). Recent studies indicated that phosphorylated BASL functions as a scaffold to recruit the MAPKKK YODA (YDA) and MPK3/6 to the cell cortex, thereby the MAPK cassette is locally concentrated so that the key stomatal factor SPEECHLESS (SPCH) is inhibited, thus the stomatal cell fate differentiation (2). However, how the BASL-YDA complex is regulated by other genetic components remains unknown. In this study, we report the AGC protein D6 PROTEIN KINASE (D6PK) family (3) as a new regulator of stomatal development and patterning in Arabidopsis. Here, we describe the stomatal defects caused by the loss-of-function and overexpression of the D6PK members in Arabidopsis. We show that D6PK co-localizes with BASL and YDA at the cell cortex, and the D6PK polarity is dependent on the BASL function. Genetic analysis suggests D6PKs function upstream of BASL-YDA complex, likely regulating the BASL protein stability. Taken together, our data suggest that the D6PK protein family regulates stomatal development and patterning by participating and regulating the BASL-YDA complex.

Introduction

Stomatal development and patterning in Arabidopsis are controlled by a genetic
pathway that is composed of several peptide ligands, receptor-like protein/kinases, a MAPK cascade, and a set of bHLH transcription factors, including the initiation factor SPEECHLESS (SPCH) (4-6). The MAPK cascade integrates intrinsic and extrinsic signals to stomatal development, involves the sequential phosphorylation and activation of a three-kinase module: the MAPK kinase kinases YDA, the MAPK kinases (MKK4/5/7/9), and the MAPK3/6 (7-9). The MAPK cascade functions downstream of receptor-like kinases genetically, the signal is then transduced to phosphorylate SPCH and affect its activity (6). BASL is a plant-specific protein that regulates stomatal patterning in stomatal lineages. In basl null mutant, the asymmetric cell division patterns are disrupted, and it generates excessive stomata clusters and small cell divisions (1). In Arabidopsis, after the meristemoid mother cell (MMC) undergoes asymmetric cell divisions, SPCH is normally maintained in the meristemoid (M) but degraded in the stomatal lineage ground cells (SLGCs) by the activity of MAPK signaling (2). This phenomenon is coincident with the dynamic turnover of BASL function. BASL is initially expressed in the nucleus and asymmetrically distributed at plasma membrane before an asymmetric cell division, while after the division, its nuclear expression is only maintained in the meristemoid, and the polarized localization at the plasma membrane is inherited to the SLGC. The polarized BASL recruits the MAPK cascade by its direct interaction with the MAPKKK YDA to enrich the MAPK signaling in the SLGC, where rapid degradation and inactivation of SPCH protein is triggered (6). In turn, the elevated MPK3/6 activities promote more BASL to be phosphorylated and to localize to the plasma membrane.
Thus, polarity protein BASL and the MAPK cascade form a positive feedback loop to regulate the stomatal cell fate determination (2, 10).

The plant-specific AGC kinase family are homologs of mammalian cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and phospholipid-dependent protein kinase C (11). Within the Arabidopsis AGC kinase family, the AGCVIII subgroup is composed of 23 kinase proteins, including the serine-threonine kinase PINOID (PID), the blue light receptors PHOTOTROPIN1 (PHOT1) and PHOT2, root growth regulators WAVY ROOT GROWTH1 (WAG1) and WAG2, D6 PROTEIN KINASEs (D6PKs), and a recent identified PROTEIN KINASE ASSOCIATED WITH BRX (PAX) protein family (11-13). These kinases regulate growth responses mainly by changing auxin transport or auxin response (3, 13-16). There are four D6PK-encoding genes in Arabidopsis, i.e. $D6PK$, $D6PKL1$, $D6PKL2$, and $D6PKL3$. The high order mutants of D6PK are defective in many aspects of developmental processes, including lateral root development, the phototropic hypocotyl bending, leaf flattening, and axillary shoot development, etc. (3, 17, 18). The D6PK protein is also known as a polarity protein and it co-localizes with the auxin efflux PIN-FORMED (PIN) proteins at the basal side of the plasma membrane in Arabidopsis roots, and D6PK phosphorylates to activate the PIN proteins in vivo and in vitro (3, 17, 18). In this study, we found that the high order mutants of the D6PK family and overexpression of D6PK both showed defects in stomatal development and patterning. In the stomatal lineage cells, D6PK co-localizes with BASL at the plasma membrane, and the D6PK polarized localization is dependent on BASL. Also, our genetic data support that D6PK
functions upstream of BASL and YDA, possibly by regulating the BASL protein stability. Thus, our results establish the roles of the D6PK protein kinase family in the regulation of stomatal development and patterning by being part of the BASL-YDA polarity complex in Arabidopsis.

Results

D6PK Regulates Stomatal Development and Patterning in Arabidopsis

The D6PK protein family is involved in multiple aspects of plant growth and development. We then asked if D6PKs function in stomatal development. We obtained the triple and quadruple T-DNA insertion mutants of D6PK family d6pk012 (d6pk;6pkl1;d6pkl2) and d6pk0123 (d6pk;d6pkl1;d6pkl2;d6pkl3) (3). We found both mutants exhibit several morphological defects, including narrow leaves, reduced plant height, and defective lateral root formation, as previously reported (3). More importantly, we detected stomatal defects in these mutants. Stomatal index (SI, the ratio of number of GCs to total epidermal cells) has been generally used as an indicator to measure stomatal production. The overall stomatal production in d6pk012 (data not shown) and d6pk0123 (SI of 23.3%, n= 3913 total epidermal cells) mutants is similar to the WT level (Col-0, SI of 23.7%, n=1628), though the mutants produce more abnormally small epidermal cells (Fig. 1G). However, the stomatal patterning in these mutants is defective, indicated by increased stomatal clusters and extra stomatal lineage cell divisions (Fig. 1A-C). The quantification data confirmed the stomatal cluster index (SCI, the ratio of clustered GC number relative to the total GC
number) is significantly increased in d6pk0123 (SCI of 21.1%, n=916 total GCs), compared to the WT (SCI of 0.0%, n=386) (Fig. 1H). These results suggest that D6PK negatively regulates stomatal production and plays an important role in stomatal patterning.

To further understand the function of D6PKs in stomatal development, we ectopically expressed D6PK gene, driven by the stomatal-specific BASL promoter (BASLpro::GFP-D6PK) and the iniquitous 35S promoter (35Spro::myc-D6PK) in Arabidopsis. Both transgenic lines generate similar stomatal defects, including stomatal clusters and abnormal cell divisions (Fig. 1D, E). Quantification indicated overexpression of myc-D6PK slightly increased stomatal production (SI of 27.1%, n=1973), compared to the WT (SI of 23.7%, n=1628) (Fig. 1G). Moreover, the stomatal patterning was obviously disrupted in myc-D6PK plants (SCI of 48.7%, n=542), compared to the WT (SCI of 0.0%, n=386) (Fig. 1H). We further found the D6PK-generated phenotype seems to depend on its kinase activity since overexpression of a kinase-inactive D6PK (myc-D6PKm, driven by the 35S promoter) failed to disrupt stomatal patterning in plants (SCI of 1.1%, n=490) (Fig. 1H). myc-D6PKm carries a K (Lysine) to E (Glutamate) amino acid substitution in the ATP-binding pocket of the kinase domain that disrupts the kinase activity (3). The increased stomatal production in myc-D6PK plants (SI of 27.1%, n=1973) was also significantly lowered in myc-D6PKm (SI of 19.6%, n=2511) (Fig. 1G). Taken together, our results suggested that, as both knocking-out and overexpressing the D6PK protein interfered stomatal patterning, a proper expression level of D6PK seemed to
be essential for normal stomatal development in Arabidopsis.

**D6PK Associates with BASL-YDA Polarity Complex**

When GFP-D6PK driven by the stomatal-specific BASL promoter was expressed in plants, we found the GFP-D6PK fusion protein is polarly localized at the cell cortex (Fig. 1D), a pattern similar to the well-known polarity protein BASL (1). The previous study showed that BASL recruits the MAPK cascade, including YDA and MPK3/6, forming a polarity complex at cell cortex to inhibit stomatal cell fate (2). We then asked if D6PK protein is also involved in the BASL-YDA polarity complex. Bimolecular fluorescence complementation (BiFC) assays indicated that D6PK associates with BASL and YDA-N domain in tobacco epidermal cells (Fig. 2A-C). Interestingly, although BASL alone is present at the cell cortex and in the nucleus, the association of cYFP-D6PK and nYFP-BASL only occurs at cell cortex region and in a polarized manner (Fig. 2B). We further crossed Arabidopsis transgenic lines of GFP-BASL and mCherry-D6PK (both driven by BASL promoter). In the progeny seedlings that harbor both constructs, GFP-BASL localizes at nucleus and polarity site of cell cortex (Fig. 2D), mCherry-D6PK only localizes at polarity site of cell cortex (Fig. 2E), and two proteins highly overlap at the polarity site (Fig. 2F). These results indicate that D6PK associates with the BASL-YDA polarity complex in plants.

**D6PK Polarity is Dependent on BASL**

The previous study reported the polarization of D6PK in root cells is regulated by the ARF GEF GNOM-mediated endosomal recycling pathway in Arabidopsis, and the plasma membrane-association of D6PK is disrupted by the ARF GEF inhibitor
Brefeldin A (BFA)-treatment (17). Similar to GFP-D6PK, mCherry-D6PK driven by
*BASL* promoter also shows polarized localization at the plasma membrane in WT (Fig.
3A, C). When expressed in the loss-of-function *basl*3 mutants, both GFP-D6PK and
mCherry-D6PK lost their polarity and plasma membrane association (Fig. 3B, D).
Both D6PK fusion proteins are internalized and form intercellular compartments in the
*basl*3 mutant background (Fig. 3B, D). These results indicate that D6PK polarized
localization at the plasma membrane requires the presence of *BASL* and the protein
function.

**D6PK Functions Upstream of BASL and May Regulate BASL Protein
Accumulation**

To study the genetic relationship between D6PK with BASL, we performed genetic
analysis using the *d6pk0123* quadruple mutant and *basl*2 mutant. The loss-of-function
*basl*-2 mutant exhibits excessive cell divisions and GC patterning defects, thus
generates a large number of small cells and stomatal clusters (Fig. 4B). The
*d6pk0123* mutant shows similar but milder phenotype (Fig. 4A). The quintuple mutant
*basl*2;*d6pk0123* showed lots of stomatal clusters and abnormal cell divisions, a
phenotype recapitulating the loss-of-function *basl*-2 mutant (Fig. 4C). Since the most
obvious stomatal phenotype in *d6pk0123* mutants is the increased number of
stomatal clusters, with the SCI increased to 21.1% (n=916 total guard cells),
compared to the SCI of 0.0% in WT (n=386) (Fig. 1H), we therefore quantified the SCI
in *basl*2 and *basl*2;*d6pk0123* mutants. The quantification data confirmed the stomatal
clustering phenotype in the progeny quintuple mutant is similar to that in *basl*2 mutant,
with the SCI of 53.2% (n=950) and 48.1% (n=555), respectively (Fig. 4D). This result indicates that \textit{D6PK} functions at the same genetic pathway with \textit{BASL}, most likely upstream of \textit{BASL}, to regulate stomatal development and patterning.

To corroborate this result, we introduced GFP-\textit{BASL} (driven by its own promoter) into the \textit{d6pk0123} quadruple mutant. The GFP-\textit{BASL} protein is expressed in the nucleus and at polarity site of cell cortex in the WT background (Fig. 4E). When expressed in the \textit{d6pk0123} mutant, the GFP-\textit{BASL} protein is also localized in the nucleus and its polarity at the plasma membrane remains unaffected (Fig. 4F). However, the GFP-\textit{BASL} protein abundance was dramatically reduced, with very few numbers of cells expressing visible GFP-\textit{BASL} in \textit{d6pk0123} mutant (1.9% in total number of 1816 epidermal cells), compared to that in WT (13.3% in total number of 669 epidermal cells) (Fig. 4F, and quantified in Fig. 4G). To figure out if the reduced GFP-\textit{BASL} accumulation was due to reduced levels of the transcripts, we performed quantitative real-time PCR analysis. Our data indicated the transcriptional level of GFP-\textit{BASL} was indistinguishable in WT and \textit{d6pk0123} background (Fig. 4H). Taken together, our result suggests D6PK protein family functions upstream of BASL, and D6PKs possibly regulate the BASL protein accumulation.

\textbf{D6PK Functions Upstream of YDA in Stomatal Differentiation}

Our data suggested D6PK associates with BASL-YDA polarity complex, we then asked the genetic relationship between \textit{D6PK} and the MAPKKK \textit{YDA} in stomatal regulation. The loss-of-function \textit{yda} mutant produces excessive stomatal clustering and cell divisions (Fig. 5B), and the \textit{d6pk012} triple mutant exhibits much milder
phenotype, with occasional stomatal clusters and more small cells (Fig. 5A). After crossing the mutations together, the quadruple mutant d6pk012;yda generates a large number of stomatal clusters and small cells, phenocopying those in yda and indicating that yda is epistatic to d6pk012 (Fig. 5C). To confirm D6PK functioning upstream of YDA, we crossed the transgenic lines mCherry-D6PK with YDA-YFP, both driven by the BASL promoter. The mCherry-D6PK line generates stomatal clusters and many small cells, similar to the GFP-D6PK line (Fig. 5D), whilst overexpression of YDA-YFP suppresses stomatal differentiation and generates lots of arrested small cells, thus very few mature GCs are formed (Fig. 5E). After the two transgenic lines are crossed, the progeny seedlings exhibit arrested small cells and almost no GC formation, a phenotype replicating that of the YDA-YFP overexpression line with the D6PK overexpression phenotype masked (Fig. 5F). Again, these results suggest that YDA-YFP functions downstream of D6PK in term of suppression of stomatal differentiation.

Discussion

The plant-specific AGCVIII D6PK protein family is a conserved protein kinase family that is involved in many developmental processes in Arabidopsis, including lateral root initiation, root gravitropism, and shoot differentiation in axillary shoots (3, 17, 18). Before this study, whether D6PKs regulate stomatal development and patterning is unknown. The high order mutants of D6PKs exhibit abnormal cell division patterns and generate excessive stomatal clusters in Arabidopsis epidermis, suggesting
potential functions of D6PKs in the regulation of stomatal development. Because of the similarity in polarized cortical localization between D6PK and the plant-specific polarity protein BASL, we found that D6PK associates with the BASL-YDA polarity complex in plant cells and its polarized localization is dependent on BASL function. Genetically, D6PKs function upstream of BASL-YDA complex in stomatal development and patterning, possibly through regulating BASL protein stability. Thus, our results identify D6PK as an upstream regulator of BASL-YDA polarity complex in Arabidopsis.

Although more evidence is required, we suspect that BASL protein stability might be regulated by D6PK-mediated protein phosphorylation. D6PK was found to phosphorylate and activate the auxin-efflux carrier PIN proteins at the plasma membrane (17). Meanwhile, the localization and function of BASL were also found closely related to its phosphorylation status. MPK3/6 phosphorylate BASL and promote its plasma membrane association, while the non-phosphorylation BASL variant (BASL_123456A) loses the plasma membrane localization and is not able to complement the loss-of-function basl2 mutant phenotype (2). Subdomain analyses of the BASL protein also suggested an intramolecular mechanism underlying cell type-specific accumulation of this polarity protein in the leaf epidermis (10). In d6pk0123 mutants, although the GFP-BASL localization pattern is not altered, its expression seems to be greatly affected, and it is not because of reduced transcriptional levels (Fig. 4F, G, H), hinting a positive contribution of D6PK to the BASL protein stability. In future, it will be interesting to test whether and how
D6PK-mediated phosphorylation may regulate the BASL protein stability and function. It is not clear why the overexpression of D6PK generates similar stomatal phenotype to loss-of-function d6pk mutants (Fig. 1B-E, G, H). This is consistent with the previous report that overexpression of D6PK results in similar developmental defects as those in the loss-of-function d6pk mutants, i.e. fewer lateral root formation (3). It is also worth noting that overexpression of the kinase-dead version of D6PK is not able to generate stomatal clustering phenotype (Fig. 1F-H). It is possible that plants need a balanced kinase activity of D6PK to achieve proper functions in a specific developmental context.

Interestingly, the functions of D6PK can be linked to another polarity protein family, four BREVIS RADIX (BRX) and BRX-like (BRXL) proteins family. In stomatal lineages, BRX polarity is dependent on BASL, and on the other hand, BASL plasma membrane association is dependent on BRX (19). In root cells, recent studies revealed that BRX and PROTEIN KINASE ASSOCIATED WITH BRX (PAX) are interacting plasma membrane-associated, both polarly localized at the rootward end of developing protophloem sieve elements (PPSEs) (13). PAX is the close homologue of D6PK, and is also named as D6PK/D6PKL-related kinase (13). PAX as D6PK may activate PIN-mediated auxin efflux, whereas BRX strongly suppresses the stimulation function of D6PK. The efficient BRX plasma membrane localization depends on PAX, but auxin negatively regulates BRX plasma membrane association and promotes PAX activity. Thus, BRX and PAX form a molecular rheostat that dynamically modulates auxin flux to maintain the intracellular auxin homeostasis in PPSE differentiation (13).
Our result identified D6PKs are involved in the BASL polarity complex in the stomatal lineage cells. Considering the close homology between D6PK and PAX, the interdependency between BASL and BRX, it will be exciting to test if the functional connection of BRX and PAX in auxin signaling is also related to the BASL-YDA-D6PK polarity module in the stomatal lineage to regulate stomatal development and patterning.

Methods

Plant materials and growth conditions

*Arabidopsis thaliana* Col-0 was used as the wild-type (WT) unless otherwise noted. The mutant and marker lines used in this study were *yda*-3 (Salk_105078), *basl*-2 (WiscDsLox264F02), *basl*-3 (SAIL_547_F11), *BASLpro::GFP*-BASL (1), and *BASLpro::YDA-YFP* (2). The *d6pk012* (3) and *d6pk0123* (3) seeds were the generous gifts of Dr. Claus Schwechheimer.

Unless otherwise noted, Arabidopsis seeds were germinated on half-strength Murashige and Skoog (MS) medium at 22°C in constant light for 6-10 days. Seedlings were then transferred to potting mix for growth in 22°C growth chambers with 16-hr light/8-hr dark cycles.

The Arabidopsis Genome Initiative (AGI) numbers under investigation in the study are: BASL (AT5G60880), YDA (AT1G63700), D6PK (At5g55910), D6PKL1 (At4g26610), D6PKL2 (At5g47750), and D6PKL3 (At3g27580).

Plasmid construction and primer designs
The LR Clonase II-based gateway cloning technology (Invitrogen) was used to generate most constructs unless otherwise noted. To generate $\text{BASLpro}::\text{GFP/mCherry-D6PK}$, the coding region of D6PK gene was first cloned from Arabidopsis cDNA (from Arabidopsis adult rosette leaves) to pENTR/D-TOPO vector (Invitrogen) and subsequently subcloned into binary destination vectors pMDC43-GFP-GW or pMDC43-mCherry-GW with the 35S promoter replaced by $\text{BASL}$ promoter (20). The coding sequence of the kinase-inactive D6PK (D6PKm) that carries a K to E amino acid substitution was amplified from a construct GST-D6PKm (3). Primers used to amplify these sequences are listed in Table S1. To make $35\text{Spro}::\text{myc-D6PK/D6PKm}$, coding sequences of D6PK/D6PKm were cloned into the binary vector pGWB618. To generate the BiFC constructs for tobacco transient expression, pENTR/D-TOPO vectors containing the coding sequence of D6PK was recombined to destination vector pCXGW (21). Plasmids were transformed into $\text{Agrobacterium tumefaciens}$ strain GV3101 for Arabidopsis transformation and tobacco leaf infiltration.

**Confocal imaging and image processing**

All confocal images were captured with a Leica TCS SP5 II confocal microscope. Unless otherwise noted, adaxial epidermis from cotyledons of 3-dpg (day-post-germination) Arabidopsis seedlings was used. Cells outlines were visualized by the propidium iodide (PI, Invitrogen) staining. Fluorescent proteins were excited at 488 nm (GFP), 514 nm (YFP), 543 nm (mCherry) and 594 nm (PI). Emissions were collected at 501-528 nm (GFP), 520-540 nm (YFP), 600-620 nm
(mCherry), and 591-636 nm (PI). Confocal images were false-colored and brightness/contrast was adjusted with Fiji ImageJ software.

**Quantitative analysis of stomatal phenotype and GFP-BASL accumulation**

For quantification analysis of stomatal phenotype, 5-dpg seedlings were stained with propodium iodide (PI, Invitrogen) and adaxial cotyledon images were captured with a Carl Zeiss Axio Scope A1 fluorescence microscope equipped with a ProgRes camera (Jenoptik). Stomatal index (SI) was calculated as the percentage of stomatal guard cell number relative to the total number of epidermal cells. Stomatal cluster index (SCI) was calculated as the percentage of clustered guard cell numbers relative to the total number of guard cells. The student’s t-test was used to determine if a difference is significant between lines.

To quantify GFP-BASL protein expression levels, frequencies of GFP-positive cells were counted for comparison. Seedlings expressing GFP-BASL in WT or d6pk0123 were stained with PI and confocal images were captured from adaxial cotyledon epidermis. The number of GFP-positive cells and total epidermal cells were counted with Fiji ImageJ software, and the ratio of GFP-positive cells among total epidermal cells were calculated. The student’s t-test was used to determine if the difference is significant between treatments.

**RNA extraction and real-time PCR**

Total RNAs were extracted from 5-dpg Arabidopsis seedlings using the RNeasy Plant Mini Kit (Qiagen). The first-strand cDNAs were synthesized by the qScript cDNA SuperMix (Quantabio) with 500 ng of total RNAs as templates.
For real-time PCR assay, reactions were prepared by an SYBR Green Master Mix kit (ThermoFisher) and conducted by the StepOnePlus Real-Time PCR System (ThermoFisher). Three technical replicates were performed for each of three biological replicates. Expression values were normalized to the reference gene ACTIN2 using the ΔCT method. Data are presented as mean ± SD. Primers used in this study were listed in Table S1.

**Transient expression in *Nicotiana benthamiana* and BiFC assay**

*Agrobacterium tumefaciens* strain GV3101 carrying binary constructs was cultured overnight in 10 mL Luria-Bertani (LB) medium with appropriate antibiotics. Agrobacterial cells were collected at 3,000 rpm for 10 min and resuspended in 10 mL of 10 mM MgCl2, followed by washing with 10 mM MgCl2, then OD600 of cell cultures were measured. For single construct transient expression assay, cell cultures were diluted to final OD600 of 0.5. For the bimolecular fluorescence complementation (BiFC) assay, cell cultures of the BiFC pairs were equally mixed with p19, followed by final dilution to OD600 at 0.5. In both assays, mixed cell cultures were then infiltrated to 4-week-old tobacco leaves. Leaf disks were excited 2-3 days after infiltration and fluorescence was checked with the confocal microscope. Three independent experiments were performed to obtain the representative images.
Figures and figure legends

**Fig. 1. D6PKs regulate stomatal development and patterning in Arabidopsis**

(A-C) Confocal images of 3-dpg adaxial side of the cotyledon epidermis in Col (A), d6pk012 (B), and d6pk0123 (C) seedlings. (D-F) DIC images of 5-dpg cotyledons to show stomatal phenotypes of BASLpro::GFP-D6PK (D), 35S::GFP-D6PK (E), and 35S::GFP-D6PKm (F) seedlings. Inset in (D) shows the representative protein localization (green), cell outlines were shown with the propidium iodide (PI) staining.

(G) Quantification of stomatal index (SI) in 5-dpg adaxial cotyledons. (H) Quantification of stomatal cluster index (SCI) in 5-dpg adaxial cotyledons. Data are mean ± SD. n=930-3334 total epidermal cells of 10-12 individual plants. *significantly different compared the WT (Col) values unless otherwise specified (Student’s t-test, *P < 0.05, **P < 0.001). n.s: not significant. Cell outlines were stained with PI. Scale bars represent 50 µm.
Fig. 2. D6PK associates with BASL-YDA polarity complex.
(A-C) Confocal images for the BiFC assay to test protein-protein interaction between D6PK and BASL, YDA-N. The expression of half YFP (YFPN) was used as negative control. (D-F) Confocal images of GFP-BASL (D, green), mCherry-D6PK (E, red) and overlay (F) localizations in 3-dpg F2 seedlings of GFP-BASL×mCherry-D6PK, both driven by BASL promoter. Overlay showing colocalization of GFP-BASL and mCherry-D6PK. Insets in (D-F) showing detailed localizations. Scale bars represent 50 µm.
Fig. 3. D6PK polarity is dependent on BASL.
(A-D) D6PK polarity at the plasma membrane is disrupted in basl3 mutant. Confocal images of 3-dpg seedlings expressing GFP-D6PK (green) and mCherry-D6PK (white), both driven by the BASL promoter, either in WT (Col) (A, C) or basl3 background (B, D). Scale bars represent 10 µm.
Fig. 4. D6PK functions upstream of BASL and may regulate BASL protein accumulation.

(A-C) Stomatal phenotype of d6pk0123 (A), basl2 (B), and basl2; d6pk0123 (C) seedlings. Confocal images were taken on the adaxial side of 3-dpg cotyledon epidermis. Cell outlines were visualized by PI staining. Scale bars represent 50 µm.

(D) Quantification of SI in seedlings of (A-C). (E-F) Confocal images showing GFP-BASL expression in Col (E) and d6pk0123 mutant (F) background. Inset in (F) showing detailed localization of GFP-BASL. GFP expression was artificially colored with green and cell outlines were visualized by PI staining. Arrows indicate weak GFP signal in d6pk0123 mutant. (G) Quantification of GFP positive cell ratio in epidermal cells in (E, F). Scale bars represent 50 µm. (H) Quantitative real-time PCR analysis of GFP-BASL expression in Col (WT) and d6pk0123 mutant background. Data are mean ± SD. *significantly different from the WT control values (Student’s t-test, *P < 0.05, **P < 0.001). n.s: not significant.
Fig. 5. D6PK functions upstream of YDA in stomatal differentiation.

(A-C) Confocal images showing stomatal phenotype of 3-dpg D6PK012 (A), YDA (B), and D6PK012;YDA (C) mutant seedlings. YDA mutant phenotype is epistatic to D6PK012 mutant phenotype. (D-F) DIC images showing stomatal phenotype of 5-dpg mCherry-D6PK (D), YDA-YFP (E), and YDA-YFP×mCherry-D6PK (F) seedlings, all driven by the BASL promoter. YDA overexpression phenotype is epistatic to D6PK overexpression phenotype. Cell outlines were visualized by PI staining. Scale bars represent 50 µm.
**Table S1. Primers used in this study**

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REFERENCES FOR CHAPTER THREE


CHAPTER FOUR

Title: The MAPK substrate MASS proteins regulate stomatal development in Arabidopsis

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This chapter is in the format of the manuscript submitted to PLOS Genetics, and is collaborated by X. Xue, C. Bian, and X. Guo.
Abstract

Stomata are specialized pores in the epidermis of the aerial parts of a plant, where stomatal guard cells close and open to regulate gas exchange with the atmosphere and restrict excessive water vapor from the plant. The production and patterning of the stomatal lineage cells in higher plants are influenced by the activities of the widely-used mitogen activated protein kinase (MAPK) signaling components. The phenotype caused by the loss-of-function mutations suggested pivotal roles of the canonical MAPK pathway in the suppression of stomatal formation and regulation of stomatal patterning in Arabidopsis (1, 2), whilst the cell type-specific manipulation of individual MAPK components revealed the existence of a positive impact on stomatal production (3). Among a large number of putative MAPK substrates in plants, the nuclear transcription factors SPEECHLESS (SPCH) and SCREAM (SCRM) are targets of MAPK 3 and 6 (MPK3/6) in the inhibition of stomatal formation (4-6). The polarity protein BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) is phosphorylated by MPK3/6 for localization and function in driving divisional asymmetries (7). Here, by functionally characterizing three MAPK SUBSTRATES IN THE STOMATAL LINEAGE (MASS) proteins, we establish that they are plasma membrane-associated, positive regulators of stomatal production. MPK6-mediated phosphorylation regulates the subcellular partition and function of MASS. Our fine-scale domain analyses identify critical subdomains of MASS2 required for specific subcellular localization and biological function, respectively. Furthermore, our data indicate that the MASS proteins may directly interact with the MAPKK Kinase
YODA (YDA) at the plasma membrane. Thus, the deeply conserved MASS proteins are tightly connected with MAPK signaling in Arabidopsis to fine-tune stomatal production and patterning, providing a functional divergence of the YDA-MPK3/6 cascade in the regulation of plant developmental processes.
**Author Summary**

Stomata surrounded by guard cells are breathing pores in the plant epidermis, where they open to allow gas exchange and close to restrict water loss. The production and patterning of stomata in the model plant Arabidopsis provide an ideal genetic and cell biological system for studying the molecular mechanisms underlying developmental program and plasticity in responding to environmental changes. The MAPK cascades are ubiquitous signaling modules in eukaryotes. They regulate diverse cellular programs by relaying extracellular signals to intracellular regulators. In the model plant Arabidopsis, MAPK 3 and 6 (MPK3/6) were found to phosphorylate several protein substrates in the nucleus and cytoplasm to regulate stomatal development and patterning. In this study, we report that a group of new MAPK substrates, the MASS proteins, function at the plasma membrane to regulate stomatal production and patterning in Arabidopsis. Thus, the output of MAPK signaling in the regulation of stomatal development is diverged by differentially localized substrates, suggesting that the concerted activities of MAPK substrates fine-tune stomatal development to ultimately improve plant adaptability to the changing environment.
Introduction

The mitogen-activated protein kinase (MAPK) cascades are central signaling pathways that regulate a wide range of cellular processes in plant growth, development and stress responses (8-10). They function downstream of the cell-surface receptors to deliver and amplify extracellular stimuli that trigger a myriad of cytoplasmic and nuclear responses (11). Stomatal development and patterning in the model plant Arabidopsis are tightly regulated by a canonical MAPK signaling cascade composed of the MAPKK kinase YODA (YDA), MAPK Kinase 4 and 5 (M KK4/5) and MAPK 3 and 6 (MPK3/6) (1-3). Mutants and genetic analyses established a pivotal role of this YDA MAPK signaling pathway in suppressing Arabidopsis stomatal production at early developmental stages (1-3). On the other hand, at later developmental stages, a positive regulation on stomatal proliferation was identified that seemed to be achieved by a differently assembled MAPK module of YDA-MKK7/9-MPK3/6 and other unknown MAPKs (3, 12).

The MAPK cascades control a diverse variety of biological processes that are achieved by the regulation of a plethora of substrates. In stomatal development, several key factors are modified and regulated by MAPKs. The stomatal lineage initiation is controlled by the bHLH transcription factors SPEECHLESS (SPCH) (13, 14) and its partners SCREAM/ICE1 (SCRM/ICE1) and SCRM2 (15). MPK3/6 phosphorylate SPCH for protein degradation, thus providing a mechanistic link to the suppression of stomatal production (4). Recent studies showed that SCRM/ICE1 is also phosphorylated by MPK3/6, so that SCRM/ICE1 protein stability was reduced in
cold tolerance and stomatal development (16). Besides these strong negative regulation of MPK3/6 signaling in the early stages of stomatal development (2, 4), a positive role of the YDA-MKK7/9-MPK3/6 at the late stages was also suggested by the stage-specific manipulation of different tiers of this MAPK cascade in Arabidopsis (3, 12). However, what substrate/s control this cell fate flip remains unknown. Previously, a few collections of putative MAPK substrate proteins were predicted by peptide library screening combined with bioinformatics analysis (17), protein-protein interaction based on yeast two-hybrid screening (18, 19), and in vivo phosphoproteomic studies (20). However, detailed functional characterization of these proteins requires significant endeavor, thus the predicted candidates have been seldomly further pursued.

The YDA-MKK4/5-MPK3/6 MAPK signaling pathway functions downstream of the plasma membrane receptor-like proteins (RLPs) and kinases (RLKs), including TOO MANY MOUTH (TMM), the ERECTA family and the Somatic Embryogenesis Receptor Kinase (SERK) family (21-25). Upstream of the MAPKKK YDA, a few regulators have been characterized for their functions in plant development, including the SHORT SUSPENSOR (SSP) receptor-associated kinase (26) and the G protein subunit Gβ in zygotic development (27), the GSK3-like BRASSINOSTEROID INSENSITIVE 2 (BIN2) kinase (28, 29) and a MAPK scaffold polarity protein BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) in stomatal asymmetric cell division (7). Their modulation of YDA’s function can be achieved by enzymatic inhibition/activation, physical scaffolding for signal specificity and
spatiotemporal restriction, etc.

Here, we characterize a new family of MAPK substrates identified from a previous large-scale screen, the three MAPK SUBSTRATES IN THE STOMATAL LINEAGE (MASS) proteins, for their biological functions in Arabidopsis stomatal development. The MASS proteins are associated with the plasma membrane where they promote stomatal formation and regulate stomatal patterning. We provide experimental evidence supporting that MAPK-mediated phosphorylation regulates the MASS subcellular localization and protein function, and in turn the MASS proteins interact with YDA at the plasma membrane, possibly suppressing YDA function. Thus, the functional connection between the MASS family and the YDA MAPK cascade provides a new angle to study how external signals through MAPKs fine-tune stomatal development at the plasma membrane.

Results

MASS proteins positively regulate stomata formation.

The previous work by Sörensson et al. (17) showed that At1g80180 is a substrate of MPK3 and MPK6. Inspired by that, we investigated its biological function in stomatal development and possible functional interaction with the core YDA MPK3/6 pathway in Arabidopsis. At1g80180 encodes a short protein (15 kD) with an unknown-of-function domain and belongs to a small family of three in the Arabidopsis genome (At1g15400 and At5g20110) (Fig 1A). We overexpressed the three genes either in the stomatal lineage cells by using a cell type-specific BASL promoter or
ubiquitously by using the CaMV 35S promoter. The results showed that all the transgenic populations produced similar stomatal phenotypes: overproliferated early stomatal lineage and guard cells in a clustered pattern (Fig 1B-D and Fig 8A). The elevated transcript levels in these overexpression lines were demonstrated by real-time PCR (Fig 1E). The phenotypes suggest that all three genes might positively regulate stomatal production, therefore are named as MAPK SUBSTRATES IN THE STOMATAL LINEAGE (MASS) genes.

To functionally characterize the three MASS genes, we analyzed their promoter activity by examination of the transcriptional reporter lines that drive the expression of nuclear YFP (nucYFP) in plants. We found that all three promoters were broadly active in the leaf epidermal cells, including young stomatal lineage cells, but mature guard cells seemed to be excluded for expression (Fig 8B). We then generated triple mutants of mass1;2;3 by crossing the three T-DNA insertional mutants (Fig 9A). The transcript levels of the three genes in mass1;2;3 mutants were assessed by real-time PCR (Fig 2E) and the data show that, while mass3 is a knock-down, mass1 and mass2 are null mutations. Quantification of stomatal production in 5-day old cotyledons suggested that the triple mutant produced lowered density of stomatal guard cells (Fig 2F), consistent with the overexpression data (Fig 1B-D and Fig 8A), supporting a positive role of the MASS genes in stomatal production. We also characterized the lower-order mutants (singles and doubles) and found that the double mutant mass 1;3, but not mass1;2 and mass2;3, produced reduced numbers of stomata, though to a less extend when compared with those of the triple mutants
(Fig 2F). None of the three single mutants showed any discernable defects in general growth and stomatal development (data not shown). Thus, our results indicated a redundantly positive role of the three MASS genes in stomatal production.

To consolidate the loss-of-function phenotypes, we deployed the CRISPR/Cas9-mediated genome-editing strategy (30) to create genetic lesions in all three MASS genes (sgRNA positions shown in Fig 9A). The wild-type plants Columbia-0 (Col) plants were transformed with the CRISPR/Cas9 construct that carried three sgRNAs, each of which should specifically target one of the three MASS genes. Two independent triple mutant lines (T3) were established (mass1;2;3-crispr#5 and #7, see the mutated sequences and genotyping data in Fig 9B, C), in which three genes were either early terminated in translation or made internal deletions (Fig 9C). Phenotypic characterization of stomatal development suggested that the CRISPR-generated mutants were similar to the T-DNA mutagenized triple mutants (Fig 2B, D), in both of which the stomatal indices were lowered to around 20% compared to that of the wild-type (23%, n=14 individual plants) (Fig 2F, G).

With respective to genetic complementation, we introduced the expression of N-terminal YFP fused MASS1 and MASS2, both driven by their endogenous promoters, into the T-DNA mass1;2;3 mutants (Fig 2C). We also introduced a CRISPR/Cas9-resistant version of mCherry-rMASS2 (rMASS2 containing nucleotide mutations in the sgRNA targeting site without changing the MASS2 amino acid sequence) into mass1;2;3-crispr#7 plants (Fig 2D). In both cases, we found that the
lowered stomatal indices in the mutants were recovered back to the wild-type levels (Fig 2F, G). In parallel, we also generated C-terminal tagged MASS proteins for complementation, but they failed to rescue the loss-of-function mutant phenotype (data not shown), were therefore abandoned for further analysis. Thus, collectively, our genetic evidence (loss-of-function and overexpression) suggested that the three MASS genes may redundantly contribute to promoting stomatal production in Arabidopsis.

**MASS functions at the plasma membrane**

By amino acid sequence analysis and prediction, no functionally annotated domains can be recognized in the three MASS proteins. To functionally characterize these novel regulators, we analyzed their subcellular localization by examining the functional translation fusions, the N-terminal fluorescent protein-tagged MASS proteins in both tobacco epidermal cells and Arabidopsis stomatal lineage cells (Fig 3A-F). The genomic regions of MASS1 and MASS3 do not contain introns, thus their genomic/coding sequences were amplified for constructing the reporter lines (CFP/YFP-MASS1/3). As three alternative splicing sites were annotated for MASS2 (Fig 9A), we amplified the genomic region flacking all three variants to generate the reporter line (CFP/YFP-MASS2g). The localization data in tobacco cells showed that the three MASS proteins were differentially distributed at the subcellular level: MASS1 and MASS2 appeared in the nucleus and at the plasma membrane, whilst MASS3 predominantly localized at the plasma membrane (Fig 3A-C). When the three genes were expressed in the stomatal lineage cells (driven by the BASL promoter), they
showed consistently differential expression patterns as in tobacco epidermal cells, with MASS1 and MASS2 dually localized in the nucleus and at the plasma membrane, while MASS3 mainly at the plasma membrane (Fig 3D-F).

Because the MASS2 protein showed expression at both subcellular locations and overexpression of it generated clustered stomatal lineage cells (Fig 3B, E, G), we tested where its biological location is, in the nucleus, at the plasma membrane, or both, to promote stomatal production. Because the MASS proteins were not predicted to contain signal peptides for apoplast secretion or transmembrane domains to span the membranes, we added a myristoylation lipid modification site to artificially tether MASS2 to the plasma membrane (31). Indeed, myr-GFP-MASS2g was found exclusively at the plasma membrane and, interestingly, overexpression of this membrane-attached version recapitulated, and even slightly enhanced, the stomatal clustering phenotypes caused by the overexpression of the wild-type protein (Fig 3H, J). On the other hand, when fused with a nuclear localization signal (NLS), YFP-NLS-MASS2g showed the anticipated nuclear-only pattern, but this version did not induce any obvious stomatal phenotypes (Fig 3I, J). The same strategies were applied to the dually localized MASS1 protein as well, and the data consistently show that plasma membrane-only but not nuclear-localized MASS1 promoted stomatal production (Fig 10A, B). Thus, collectively, our data suggested that the positive regulation of the MASS proteins in stomatal development arises from the plasma membrane pool, but not from the nuclear pool.

**Fine-scale analysis of the MASS2 subdomains**
As the MASS proteins do not contain transmembrane domains, their association with the plasma membrane might be achieved by protein-protein or protein-lipid interactions in plant cells. To characterize the subdomains, we aligned the three MASS proteins. Because MASS2 has three splicing variants, we first examined their subcellular localization (N-terminal GFP fusions driven by the BASL promoter) and found that all of them showed the typical dual localization (Fig 11B). MASS2.3, as the longest one (Fig 11A) was used as a representing member to align with MASS1 and 3 for subdomain analysis (Fig 11C). The three MASS proteins show high similarity at the two terminal regions but are not conserved in the middle (Fig 4A and Fig 11C). We thus split MASS2.3 into halves to make GFP-tagged MASS2.3_N76 and MASS2.3_C72, both driven by the BASL promoter (Fig 4A). The results showed that both truncations failed to localize correctly. The first half GFP-MASS2.3_N76 lost the distinct localization in the nucleus, whereas the second half GFP-MASS2.3_C72 lost the plasma membrane localization (Fig 4B-D). To further narrow down the critical segments for specific localization at the plasma membrane and in the nucleus, respectively, we deleted the conserved regions to create GFP-MASS2.3_Δ29N (the N-terminal 29 amino acids deleted), GFP-MASS2_Δ13C and GFP-MASS2_Δ25C (the C-terminal 13 and 25 amino acids deleted, respectively) (Fig 4A). The subcellular localization data clearly demonstrated that the N-terminal 29-aa promotes the plasma membrane-association and the C-terminal 13-aa determines the nuclear accumulation (Fig 4E, F, G). Interestingly, the plasma membrane-only GFP-MASS2_Δ13C promoted stomata production and clustering, a phenotype
resembling that of the full-length MASS2 overexpression (Fig 4H, I). However, the further shortened MASS2.3_Δ25C, albeit successfully localized to the plasma membrane, failed to induce stomatal overproduction (Fig 4J). In addition, none of the other shortened versions were sufficient to trigger this phenotype (Fig 11D). The results suggested the small region between MASS2.3_Δ25C and GFP-MASS2_Δ13C is critical for the protein function in promoting stomatal development. That would explain why the MASS2.3_N76, MASS2.3_C72 and MASS2.3_Δ29N fail to promote stomata formation (Fig 11D). The reason is these truncated versions lack either plasma membrane association part or critical function region. Taken together, we established that the MASS proteins promote stomatal formation at the plasma membrane and defined three specific regions in MASS2.3 that are important for nuclear accumulation, plasma membrane association, and the biological function at the plasma membrane, respectively (Fig 4A).

**MPK6-mediated phosphorylation regulates MASS localization and function**

Sörensson et al. (2012) previously demonstrated that MASS1 is phosphorylated by MPK6 in vitro kinase assays (17). We further tested MASS2 recombinant proteins. Because one of the splicing variants, MASS2.2, is most similar to MASS1, we purified the MASS2.2 recombinant proteins and found that MASS2.2 was phosphorylated by in vitro constitutively active M KK5 (MKK5\textsuperscript{DD})-activated MPK6 (Fig 5A). In parallel, mutating the serine residue (S107) in the conserved MAPK-substrate PPSP motif to alanine abolished the MPK6-mediated phosphorylation of MASS2.2 (Fig 5A), supporting that MASS2 is phosphorylated by MPK6 at the S107 site.
Phosphorylation may alter protein subcellular localization. We manipulated the phosphorylation site of MASS1 (S105) by generating a phospho-deficient version MASS1$_{S^{105}A}$ and a phospho-mimicking version MASS1$_{S^{105}D}$, respectively. By examining the YFP-tagged proteins, we found that phosphorylation status is influential to MASS1 subcellular distribution because neither MASS1$_{S^{105}A}$ nor MASS1$_{S^{105}D}$ showed robust plasma membrane-association, but both more abundantly accumulated in the nucleus (Fig 5B and quantification in Fig 5C, n=50 cells). With respective to the MASS2 localization, because we established that MASS2.2 was phosphorylated by MPK6 in vitro (Fig 5A), we assessed the localization pattern of YFP-MASS2.2$_{S^{107}A}$ and YFP-MASS2.2$_{S^{107}D}$ in the stomatal lineage cells. Consistently, the fluorescence intensity profiling results demonstrated that both versions showed reduced abundance at the plasma membrane but elevated accumulation in the nucleus (Fig 5D). Taken together, our data suggested that MAPK-mediated phosphorylation is important for the MASS proteins to localize robustly at the plasma membrane. The failure of YFP-MASS2.2$_{S^{107}D}$ to localized robustly at the plasma membrane was not anticipated, but suggesting that dephosphorylation might be equally important for correct localization pattern of the proteins.

To further assess the impact of protein phosphorylation on their biological functions, we first overexpressed YFP-MASS2.2, MASS2.2$_{S^{107}A}$, and MASS2.2$_{S^{107}D}$ in the stomatal lineage cells by using the BASL promoter. Our results show that MASS2.2 did not seem to function as effectively as MASS2g in triggering stomatal clusters and neither of the mis-localized phospho-variants produced significant stomatal
phenotypes (Fig 5E). Considering the functional location of MASS appeared to be at the plasma membrane, we modified MASS2.2 and the other two variants with the myristoylation site. When tethered to the plasma membrane, myr-GFP-MASS2.2 induced stronger stomatal clustering but, in contrast, neither myr-GFP-MASS2.2$^{S107A}$ nor myr-GFP-MASS2.2$^{S107D}$ could function at a comparable level (Fig 5F). Thus, we concluded that MAPK-mediate phosphorylation not only regulates the subcellular localization of the MASS proteins, it also important for their biology functions at the plasma membrane in stomatal development.

**MASS interacts with the MAPKK Kinase YDA**

The *mass* triple mutants often showed a cotyledon-fusion phenotype (data not shown), to certain extent resembling that of a plant expressing the constitutively active MAPKKK YDA (CA-yda or YDA$^{CA}$, (1)). In addition, similar to the MASS proteins, YDA is also a peripheral membrane protein in plant cells (7). To test whether the MASS genes are functionally connected to YDA, we first examined the physical interaction between MASS2 with YDA. Indeed, positive protein-protein interactions were detected between YDA and MASS2 based on our *in vitro* pull-down and yeast two-hybrid assays (Fig 6A, B). To test their interaction in plant cells, we assayed the kinase inactive version of YDA (YDA$^{KI}$ with one point mutation K429R, (3)) because overexpression of the catalytically active enzyme often cause cell death in tobacco (32). In the Bimolecular fluorescence complementation (BiFC) assay in tobacco epidermal cells, the recovered split YFP signals supported that all three MASS proteins may physically interact with YDA$^{KI}$ in plant cells and at the plasma membrane.
(Fig 6C and Fig 12A). In Arabidopsis stomatal lineage cells, we co-expressed mCherry-MASS2 and YDA\textsuperscript{KI}-YFP (driven by the SPCH promoter) and the two proteins co-existed at the plasma membrane. Furthermore, Z-projected confocal images showed that MASS2 forms cortical punctate that overlap with a portion of YDA\textsuperscript{KI}-accumulating dots at the plasma membrane (Fig 6D), though the properties of which have not been characterized yet. Taken together, our data suggested that the MASS proteins might function through their physical interaction with YDA at the plasma membrane.

Based on the phenotypes shown in the loss-of-function and overexpression plants, we hypothesized that the MASS family might promote stomatal production through suppressing the YDA MPK3/6 signal pathway, possibly via directly interacting with YDA. To examine the genetic interaction, we overexpressed CFP-MASS2 in plants expressing YDA\textsuperscript{CA}-YFP (constitutively active YDA driven by the stomatal lineage-specific SPCH promoter). While YDA\textsuperscript{CA} suppresses stomatal differentiation (Fig 7A and (1)), interestingly, the introgression of CFP-MASS2 suppressed the YDA\textsuperscript{CA}-induced phenotype by restoring the formation of stomatal lineage cells (Fig 7A). At the plasma membrane, the receptor-like kinases ER and SERK could also possibly interact with MASS. However, we did not detect physical interaction between MASS2 and the ER kinase domain in the yeast-two hybrid (data not shown). Also, when MASS2 overexpression was introduced into er mutants, an additive stomatal phenotype was observed (Fig 12 B-D), suggesting that the MASS function does not seem to rely on the presence of the ER receptor. The plasma membrane-localized
polarity protein BASL was also examined in MASS2 overexpression plants but no discernable changes in GFP-BASL localization were noticed (Fig 12E). Collectively, our results supported that the positive role of MASS in stomatal production might be achieved by physical interaction with YDA thus the suppression of the MAPK signaling pathway in Arabidopsis (Fig 7B).

**Discussion**

MAPK cascades convert and amplify environmental and developmental cues into adapted intracellular responses. Their functions are particularly important for signal transduction in sessile plants that are incapable of escaping from a stressing environment. With a large number of potential kinase-substrate relationships of plant MAPKs revealed by *in vitro* and *in vivo* strategies (17, 20), most putative MAPK substrates remained functionally elusive. Plant MAPKs usually are expressed in the cytosol and/or nucleus, therefore their substrates at different subcellular localizations are thought to largely determine signal specificity and the spatiotemporal dynamics of MAPK signaling in a biological process (33). In Arabidopsis stomatal development, SPCH and SCRM/ICE1 are nuclear transcription factors targeted by MPK3/6 for protein degradation (4, 5, 16), so that stomatal formation can be modulated by MAPK upstream signals. The polarity protein BASL in the regulation of stomatal asymmetric cell division is phosphorylated by MPK3/6 for its localization at the cell cortex where BASL functions as a MAPK scaffold protein to locally concentrate the YDA MAPK signaling to differentiate daughter cell fates (7, 34). In this study, we established the
functions of a newly identified MAPK substrate family, the MASS proteins, in the regulation of stomatal development and patterning in Arabidopsis. Phenotypic analysis of the loss-of-function mutants suggested that MASS functions to promote stomatal production and the overexpression phenotype revealed an additional role of MASS in cell-cell communication and stomatal patterning (Fig 1 and Fig 8).

The dual localization of the MASS proteins

The subcellular localization revealed by the fluorescent protein-tagged MASS proteins suggested that MASS1 and MASS2 are dually localized to the nucleus and at the plasma membrane, with the exception of MASS3 that only appeared at the plasma membrane (Fig 3). But overexpression of this plasma membrane-only MASS3 induced comparable stomatal phenotypes as those generated by overexpression of MASS1, MASS2, and their plasma membrane-only, myristoylated versions (Fig 1 and Fig 3), consistently suggesting the biological activities of all three MASS proteins occur at the plasma membrane.

Why doesn’t MASS3 localize into the nucleus? This probably can be explained by its protein sequence, in particular at the very N-terminal side (Fig 11C). Based on our data of domain analyses of MASS2.3, the nuclear localization-determining fragment has been narrowed down to the last 13 amino acids (Fig 11C). Interestingly, this fragment contains a 5 amino acid-long, basic residue-rich motif (KRRSR) that is fully conserved in MASS1 but divergent in MASS3 (SGGST). Considering that MASS1 and MASS2 both are dually localized and MASS3 is excluded from the nucleus, we suspect that the lack of this conserved K/R-rich motif, possibly a nuclear localization
signal (NLS) that could mediate the interaction with importins for nucleocytoplasmic transport (35), results in the failure of MASS3 to express in the nucleus.

With regards to the plasma membrane localization of MASS proteins, the highly conserved N-terminal regions aligned with the first 29 amino acids of MASS2.3 seemed to be required, though no obvious features, e.g. high hydrophobicity, lipid modification, etc., could be recognized to explain the mechanisms for this localization. In parallel, our work also suggested that MAPK-mediated phosphorylation of the highly conserved P-P-S-P-R/K sites (Fig 11C) might be required for MASS to localize and function at the plasma membrane. Future work on the identification of MASS protein-protein and/or protein-lipid interactions, in particularly related to the second K/R-rich functional domain (Fig 11C, PPGKKVNPRKRPP in MASS2.3), is anticipated to bring more insights to explain how these peripheral membrane proteins are localized and possibly translocated between different subcellular locations.

**Possible functions of MASS at different subcellular localizations**

We findings show that, interestingly, the localization pattern and functional fashion of the MASS proteins, to some extent, mimics those of the polarity protein BASL (36). For example, both are membrane-associated proteins that appear to be dynamically distributed between the nucleus and the plasma membrane (Fig 3A-3F) (34). Although MASS is not polarized, both proteins function at the plasma membrane but not in the nucleus (36). Also, both proteins are regulated by MAPK-mediated phosphorylation to achieve their subcellular localization and biological function (Fig 5A-5F) (7). In addition, both proteins appear to interact with the MAPKKK YDA at the
cell cortical region. Base on the data we collected, we propose that, the nuclear pool of MASS proteins, similar to that of BASL, might serve as a storage form that can be quickly targeted by MAPK signaling and redistributed to the target locations, without synthesizing new proteins, to respond to external stimuli. With regards to MASS3 that is not expressed in the nucleus, the cytoplasm region can be a buffering zone. This possibility can be further tested by deletion of the N-terminal 29 amino acids and mutating the conserved phosphorylation site.

Our complementation data supported that the MASS proteins promote stomatal production at the plasma membrane. Then, what is the molecular mechanism for MASS to function there? We provided evidence that MASS proteins may interact with the MAPKKK YDA at the cell periphery, where MASS is hypothesized to negatively impact on the MAPK signaling cascade that suppresses the protein abundance of the key stomatal factor SPCH in production and proliferation of the lineage cells (2, 4).

Also, we noted that one striking phenotype caused by MASS overexpression was clustered stomata, a phenotype reflecting defective cell-cell communication. In most dicot leaves, stomata are spaced out by at least one nonstomatal epidermal cells to followed the “one-cell-spacing” rule needed for efficient guard cell activity in gas exchange (37, 38). To enforce this patterning rule, it was hypothesized that the developing guard cells release positional signals, e.g. the peptide ligand EPF1 (39), that are perceived by the cell surface receptors, e.g. the receptor-like protein TMM (22) and the receptor-like kinase ERL1 (21, 40) with the SERK co-receptors (23), in the neighboring cells, so that the new divisions are reoriented to prevent direct stomatal
contact from occurring (41). In addition, downstream of the ligand-receptor signaling, defective YDA MAPK activities also led to the failures in enforcing the one-cell-spacing rule (1, 2). Therefore, it is possible that the elevated expression levels of MASS at the plasma membrane may directly or indirectly alleviate the ligand-receptor signaling and/or the YDA MAPK cascade activities in the regulation of division reorientation. An expanded survey for the MASS proteins to physically interact with the individual ER and SERK family member would be necessary to test this connection. Alternatively, a novel functional connection between MASS and MKK7/9 may exist. A surprising role of mitochondria localized MKK7 in specifically promoting stomatal overproduction and clustering at late stomatal stages was detected in Arabidopsis (3, 12). Whether MASS functions to promote MKK7/9 signaling can be tested by genetic and biochemical strategies in future studies.

The MASS genes, plant-specific and deeply conserved in early land plants.

Through our sequence comparison and domain analysis, two conserved domains were recognized in the MASS protein family, including one segment at the N-terminus determining localization at the plasma membrane and another one at the C-terminus, containing the K/R-rich motif required for function and the putative MAPK phosphorylation site PPSP (Fig 11C). We aligned three MASS proteins with 40 orthologs that can be identified in land plants (embryophytes) to generate the phylogenetic tree (Fig 13). In the fern Selaginella moellendorffii, liverworts Marchantia polymorpha, as well as conifer Picea sitchensis, only one MASS-like (MASS-L) protein with the conserved N-terminal domain can be identified in their respective
genome, suggesting their localization at the plasma membrane might be an ancient feature.

Interestingly, the typical MASS structure containing the two conserved domains appeared in the earliest flowering plant Amborella trichopoda, AmtMASS, and then the family members expand along the evolution of angiosperms, e.g. 7 MASS genes in maize (Fig 13). In addition, based on the phylogenetic assay, we found that MASS3 is more similar to the ancient full-length form, AmtMASS. We suspect that the full-length MASS might be coopted an ancient form (MASS-L) and include additional C-terminal MAPK site and the functional domain to act as a MAPK substrate. Thus, the combined features of the MASS proteins enable them as MAPK-responding regulators for plants to adapt to the environmental changes.

Materials and Methods

- Gene accession numbers

MASS1 (At1g80180), MASS2 (At1g15400), MASS3 (At5g20100), YDA (AT1G63700)

- Plant materials, mutants and transgenic lines

The Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type. In general, Arabidopsis and tobacco Nicotiana benthamiana plants were grown at 22°C in long days (16 h light/8 h dark). The T-DNA insertional lines mass1 (GABI_902G09), mass2 (SALK_061905), mass3 (SALK_039099) were obtained from Arabidopsis Biological Resource Center (ABRC). The GFP-BASL marker line were described previously (36).
• **Plasmid construction and plant transformation**

In general, the LR Clonase II (Invitrogen)-based gateway cloning technology was used for vector construction. To generate point mutations, the plasmid pENTR/D-TOPO carrying the MASS genomic or coding regions were used as template and specific site mutations were introduced through a QuickChange II XL Site-Directed Mutagenesis Kit (Strategene). The entry clones were then recombined into pMDC43 (the original 35S promoter was replaced by BASL promoter) and pH35CG to make BASLp::GFP/YFP/mCherry-MASS and 35S::CFP-MASS, respectively. Then, BASL promoter were replaced by MASS promoter through Pmel and KpnI sites to generate MASS1pro::YFP-MASS1 and MASS2pro::YFP-MASS2. The pENTR/D-TOPO carrying MASS promoters were recombined into pBGYN to make MASSp::nucYFP.

To create CRISPR/Cas9-mediated mutagenesis in Arabidopsis, we adopted the system described in (30). By following the instructions, the oligos MASS1-CRI-F and MASS1-CRI-R were phosphorylated by T4 PNK (NEB) and annealed in a thermocycler, followed by ligation into the BbsI site of pAtU6-sgRNA-pAtUBQ-Cas9. Then, the chimeric U6-MASS1-Cas9 cassette was cloned into pCambia 2300 through HindIII and EcoRI sites to obtain 2300/crispr_mass1. U6-MASS3 was amplified by PCR and inserted into 2300/crispr_mass1 through KpnI and EcoRI sites to generate 2300/crispr_mass1;3. Finally, by using the same strategies, U6-MASS2 were inserted at EcoRI site to create the construct 2300/crispr_mass1;3;2 to knock-out the three members in the family. The crispr resistant MASS2 version (rMASS2) was generated
through two rounds of PCR to introduce synonymous mutations in the Cas9-gRNA targeting site. Primers were listed in Table S1.

Plasmids were transformed into *Agrobacterium tumefaciens* GV3101, which delivers the desired DNA pieces into Arabidopsis or tobacco cells. *Arabidopsis* plants were transformed with the standard floral dipping method (42, 43) and transgenic seeds were subjected to antibiotic selection. Tobacco cells were infiltrated by the method described in (44). *A. tumefaciens* cells harboring 35S::CFP-MASS1/2/3 were infiltrated into *N. benthamiana* leaves and after 3 days, the leaf epidermal cells were observed under confocal microscope Leica SP5.

- **Plant cell imaging and image processing**

Confocal images of plant cells expressing fluorescence-tagged proteins were taken by a Leica SP5 confocal microscope. 3-dpg (day-post-germination) adaxial cotyledons of Arabidopsis were captured. Cell peripheries were visualized with propidium iodide (PI, Invitrogen). Fluorescent proteins were excited at 488 nm (GFP), 514 nm (YFP) and 594nm (PI). Emissions were collected at 500-528 nm (GFP), 520-540nm (YFP), and 620-640 nm (PI). The confocal images were adjusted using either Adobe Photoshop CS5.1 or ImageJ (Fiji). The fluorescence intensity was measured by ImageJ (Fiji) and the pixel values were export into Excel to generate the histogram graphs.

- **Quantitative and statistical analysis of stomatal phenotypes in *Arabidopsis***

The adaxial cotyledons from 5-dpg seedlings were stained with PI and imaged were
captured using a Carl Zeiss Axio Scope A1 fluorescence microscope equipped with a ProgRes MF CCD camera (Jenoptik). Stomata index (SI) was calculated as the stomata number versus the total number of epidermal cells. Clustered stomata index was calculated as the percentage of the number of clustered stomata over the total number of stomata. Stomatal clusters in Figure 1C were counted on the adaxial surface of the 10-dpg cotyledons.

- **Real-time PCR**

Total RNAs were extracted from 3-dpg seedlings using an RNaseasy Plant Mini Kit (Qiagen). The first-strand cDNAs were synthesized by the SuperScripTM First-Strand Synthesis System (Invitrogen) with 2µg of total RNAs as template in a total volume of 20 µl. The fragments of interest were amplified by sequence-specific primers (see Table S1). Real-time PCR was performed with a SYBR Green Master Mix kit (Applied Biosystems) and amplification was monitored on a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene expression levels were normalized to the reference gene (ACTIN2) expression using the ΔCT method. Data are presented as mean ± SD.

- **Protein–protein interaction assays**

The yeast two-hybrid assay was performed using the Matchmaker GAL4 Two-Hybrid System according to the manufacturer’s manual (Clontech). MASS2 were inserted into pGADT7 and YDA was inserted into pGBK7T7, respectively. Plasmids were transferred into the yeast strain AH109 (Clontech) by the LiCl-PEG method. The interactions were tested on SD/-Leu/-Trp/-His plates supplemented with 5 mM
3-amino-1,2,4,-triazole (3-AT). Three independent clones for each transformation were tested.

- **Pull-down assay**

The CDS fragments of MASS2 and YDA were cloned into pET28a or pMAL-c2x for *E.coli* expression of His- or MBP-tagged proteins, respectively. Constructs were introduced into BL21 (DE3) cells for recombinant proteins expression. The recombinant His-tagged MASS2 and MBP-tagged YDA were purified using Ni-NTA agarose (QIAGEN) or Amylose Resin (New England Biolabs), respectively, according to the manufacturer's protocol. For pull-down assays, 3 mg of MBP-YDA fusion protein was incubated with Amylose Resin at 4°C for 2 h, the MBP tag was used as a negative control. The beads were cleaned with washing buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 150 mM NaCl, and 1 mM DTT) for five times. Then the beads were incubated with 5 mg of His-MASS2 at 4°C for 2 h. Wash beads five times with washing buffer. Western blot was used to detect the SDS-PAGE separation results of pulled-down mixtures in nitrocellulose membrane with anti-His antibody (Cell Signaling Technology) and anti-MBP antibody (New England Biolabs).

**Acknowledgements**

We appreciate helpful advice and discussion with Dr. Huiling Xue (Shenyang Agricultural University) on the phylogenic analysis of the MASS protein family. We thank the ABRC stock center for providing the T-DNA insertional lines. Research
programs in the Dong lab are supported by the National Institute of Health R01GM109080, R35GM131827 to J.D. X.X. was supported by the Charles and Johanna Busch Fellowship from Rutgers. C.B. was supported by fellowships from the Chinese Scholar Council and Rutgers University.
Figures and Figure Legends:

(Figures)

A) Phylogenetic tree of Arabidopsis MASS family. (B) Stomatal phenotype of MASS overexpression lines. Confocal images of 3-dpg adaxial epidermis of cotyledons in WT (Col) and GFP-MASS1, GFP-MASS2g, and GFP-MASS3 seedlings, all driven by BASL promoter. Cell outlines were stained with propidium iodide (PI). Brackets indicate stomatal clusters. Scale bar represents 50 µm. (C) Quantification of numbers of stomatal clusters per cotyledon of 10-dpg seedlings in (B). (D) Quantification of stomatal index (SI) in 5-dpg adaxial cotyledons of seedlings in (B). (E) Quantitative real-time PCR analysis of MASS1/2/3 expressions in WT and GFP-MASS transgenic lines in (B). Data are mean ± SD. *significantly different compared the WT (Col) values unless otherwise specified (Student’s t-test, *P < 0.05, **P < 0.001). n.s: not significant.

(A) Phylogenetic tree of Arabidopsis MASS family. (B) Stomatal phenotype of MASS overexpression lines. Confocal images of 3-dpg adaxial epidermis of cotyledons in WT (Col) and GFP-MASS1, GFP-MASS2g, and GFP-MASS3 seedlings, all driven by BASL promoter. Cell outlines were stained with propidium iodide (PI). Brackets indicate stomatal clusters. Scale bar represents 50 µm. (C) Quantification of numbers of stomatal clusters per cotyledon of 10-dpg seedlings in (B). (D) Quantification of stomatal index (SI) in 5-dpg adaxial cotyledons of seedlings in (B). (E) Quantitative real-time PCR analysis of MASS1/2/3 expressions in WT and GFP-MASS transgenic lines in (B). Data are mean ± SD. *significantly different compared the WT (Col) values unless otherwise specified (Student’s t-test, *P < 0.05, **P < 0.001). n.s: not significant.
Fig 2. MASS positively regulates stomata formation

(A) Confocal images of 3-dpg adaxial side of the cotyledon epidermis in WT (Col) and mass1;2;3 mutant seedlings. (B) DIC images of 5-dpg cotyledons in WT and mass1;2;3-crispr #5 seedlings. (C) Confocal images of 3-dpg adaxial side of the cotyledon epidermis in complementation lines MASS1p::YFP-MASS1 and MASS2p::YFP-MASS2 in mass1;2;3 background. (D) DIC images of 5-dpg cotyledons in mass1;2;3-crispr #7 and complementation seedlings. (E) Quantitative real-time PCR analysis of MASS1/2/3 expressions in WT and mass1;2;3 mutant. (F) Quantification of SI in 5-dpg adaxial cotyledons of T-DNA double and triple mutants. (G) Quantification of SI in 5-dpg adaxial cotyledons of mass1;2;3-crispr #7 mutant and two complementation lines. Data are mean ± SD. *significantly different compared the WT (Col) values unless otherwise specified (Student’s t-test, *P < 0.05, **P < 0.001). n.s: not significant. Cell outlines in (A-D) were stained with PI. Guard cells in (B, D) were highlighted in pink for visualization. Scale bars represent 50 µm in (A-D).
Fig 3. MASS functions at the plasma membrane

(A-C) Localization of MASS proteins in tobacco leaves. Confocal images of tobacco epidermis transiently expressed with CFP-MASS1 (A), CFP-MASS2g (B), and CFP-MASS3 (C), all driven by 35S promoter. Scale bar represents 50 µm. (D-F) Localization of MASS proteins in Arabidopsis leaves. Confocal images of 3-dpg adaxial side of the cotyledon epidermis expressing YFP-MASS1 (D), YFP-MASS2g (E), and YFP-MASS3 (F), all driven by BASL promoter. Scale bar represents 10 µm. (G-I) Stomatal phenotype triggered by modified MASS2 proteins. Confocal images of 3-dpg adaxial cotyledon epidermis in plants expressing GFP-MASS2g (G), myr-GFP-MASS2g (H), and YFP-NLS-MASS2g (I), all driven by BASL promoter. Scale bar represents 50 µm. Brackets indicate stomatal clusters and abnormal cell divisions. (J) Index of clustered stomata in 5-dpg adaxial cotyledons of seedlings in (G-I). Cell outlines in (G-I) were stained with PI. Data are mean ± SD. *significantly different compared the WT (Col) values unless otherwise specified (Student’s t-test, *P < 0.05, **P < 0.001). n.s: not significant.
Fig 4. Fine-scale analysis of the MASS2 subdomains

(A) Diagram of MASS2.3 protein and proposed functions of subdomains. Brackets highlight the N-terminal subdomain that mediates plasma membrane association, and subdomains in the C-terminal that mediate its nuclear accumulation and are critical for its function. (B-G) Confocal images of 3-dpg adaxial side of the cotyledon epidermis showing localization of GFP-fused MASS2.3 with different truncations. (H-J) Confocal images of 3-dpg adaxial side of the cotyledon epidermis showing stomatal phenotype of MASS2.3 full-length and truncated proteins. Brackets indicate stomatal clusters and abnormal cell divisions. Cell outlines in (H-J) were stained with PI. Scale bar represents 20 µm in (B-G) and 50 µm in (H-J).
Fig 5. MPK6-mediated phosphorylation regulates MASS localization and function

(A) In vitro kinase assay showing MKKsDD-activated MPK6 phosphorylation of MASS2.2. (B) Upper panel showing localization of YFP-tagged MASS1 and MASS1 phosphor-variants. Lower panel shows the YFP intensity corresponding to the upper image. Red arrows indicate YFP signal at plasma membrane. Scale bar represents 10 µm. (C) Quantification of YFP intensity in different subcellular portion of fusion proteins shown in (B). (D) Upper panel, confocal images showing localization of YFP-tagged MASS2.2 and MASS2.2 phosphor-variants. Lower panel, intensity of YFP signals. Red arrows indicate YFP signals at plasma membrane. Scale bar represents 10 µm. (E, F) Quantification of clustered stomata index in 5-dpg adaxial cotyledons of YFP-tagged MASS2.2 and phosphor-variant seedlings (E) and myristoylated GFP-tagged MASS2.2 and phosphor-variant seedlings (F). Data are mean ± SD. *significantly different compared with the WT (Col) values unless otherwise specified (Student’s t-test, *P < 0.05, **P < 0.001). n.s: not significant.
**Fig 6. MASS2 interacts with YDA**

(A) Yeast two-hybrid assay for MASS2.2 with YDA. The BD and AD empty vectors were used as negative control. Interaction tests are shown on the medium supplemented with -Leu-Trp-His. (B) *In vitro* pull-down assays using recombinant proteins, MBP-YDA and His-MASS2. MBP- was used as negative control. Immunoblots were visualized by anti-His and anti-MBP. (C) BiFC assays to test the interaction between YDA\(^{K1}\) and MASS2g in tobacco leaf epidermis. The expression of half YFPs (YFP\(^{N}\) and YFP\(^{C}\)) were used as negative controls. Scale bar represents 50 µm. (D) Confocal images showing co-localization of BASLp::mCherry-MASS2g with SPCHp::YDA\(^{K1}\)-YFP in Arabidopsis cotyledons. Bottom panels showing detailed localizations. White arrows indicate co-localization of two proteins. Scale bar represents 50 µm in upper panel and 10 µm in lower panel.
Fig 7. Proposed MASS function in stomatal development

(A) DIC images of 5-dpg seedlings showing the stomatal phenotype of 35S::CFP-MASS2g, SPCHp::YDA<sup>CA</sup>-YFP, and the progeny of these two transgenic lines. The MASS2g overexpression releases the suppression of stomatal production by YDA<sup>CA</sup>. Scale bar represents 50 µm. (B) Proposed working model for MASS proteins: MPK3/6 phosphorylate MASS proteins and promote their plasma membrane association; MASS proteins interact with YDA and possibly suppress its protein function; then the inhibition on stomatal production is alleviated.
Fig 8. MASS overexpression phenotype and expression pattern
(A) Stomatal phenotype of MASS overexpression lines. Confocal images of 7-dpg adaxial side of the cotyledon epidermis in WT (Col) and CFP-MASS1, CFP-MASS2g, and CFP-MASS3 seedlings, all driven by 35S promoter. Brackets indicate stomatal clusters. Scale bar represents 50 µm. (B) Expression pattern of MASS1/2/3 genes. Confocal images showing expression of nuclear YFP driven by promoters of MASS1/2/3. Cell outlines were stained with PI. The inset showing detailed expression pattern. Scale bar represents 50 µm.
Fig 9. Mutations in MASS genes
(A) Diagram of gene structures of MASS genes and splicing variants. (B) Genotyping of CRISPR lines. DNA sequence alignments showing corresponding MASS gene editing in mass1;2;3-crispr #5 and #7 lines. PAM sequences were outlined with blue
boxes, sgRNAs were underlined with red. (C) Genotyping PCR showing a long deletion in MASS1 in T3 of mass1;2;3-crispr #7 mutant.
Fig 10. MASS1 functions at plasma membrane in stomatal lineages
(A, B) Confocal images showing stomatal phenotype in plasma membrane-localized (A) and nuclear-localized (B) GFP-MASS1 seedlings, both driven by BASL promoter. Green: GFP signals, magenta: cell outlines stained with PI. Left panels show protein localization, right panels show the overlay of green and magenta. White brackets indicate stomatal clusters and abnormal cell divisions. Scale bar represents 50 µm.
Fig 11. Subdomain analysis of MASS proteins
(A) Amino acid alignment of MASS2 splicing variants. (B) Confocal images showing the detailed localization of GFP-tagged MASS2g, MASS2.1, MASS2.2, and MASS2.3, all driven by BASL promoter. Scale bar represents 10 µm. (C) Amino acid alignment of MASS1, MASS2.3, and MASS3 and predicted subdomains required for particular functions. The deleted amino acids to make GFP-MASS2 truncation versions were outlined with designated colors. (D) Confocal images of 3-dpg adaxial side of the
cotyledon epidermis showing localization and stomatal phenotype of truncated MASS2.3 proteins. Cell outlines were stained with PI. Scale bar represents 20 μm.
Fig 12. Genetic relationship between MASS proteins and other stomatal regulators

(A) Confocal images of BiFC assays to test for interactions between YDA\textsuperscript{KI} and MASS1/3 in tobacco leaf epidermis. The expression of half YFP (cYFP) was used as negative control. Scale bar represents 50 µm. (B-D) Genetic test between \textit{er105} and GFP-MASS2g overexpression line, driven by \textit{BASL} promoter. Cell outlines were stained with PI. (E) Confocal images showing localization of Venus-BASL and mCherry-MASS2g, both driven by \textit{BASL} promoter. Scale bar represents 50 µm in (B-E).
Fig 13. Phylogenetic tree of the MASS family
The protein sequences of the three Arabidopsis MASS proteins were compared with those of 40 orthologs obtained from GenBank, representing embryophyta that includes, lycophyte, gymnosperm and angiosperms. Phylogenetic tree was constructed by the program MEGA6 (45), using the neighbour-joining method. The reliability of the phylogenetic tree was evaluated by bootstrapping of 1000 replications.
**Table S1. Primers used in this study**

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| MASS2 variant resistant to sgRNA targeting | M15400-R | TGTGAACGTCACTCATTGGTTTAGGTTTTCG
|                                      | M15400-F | ACCAATGAGTGAGTCAAGTGAAACAGGTCA
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31. Magee T & Seabra MC (2005) Fatty acylation and prenylation of proteins:


CHAPTER FIVE

CONCLUSION AND FUTURE PERSPECTIVES

Land plants require the formation of stomatal guard cells to allow CO2 to come into the internal tissue for photosynthesis, a process immensely beneficial for the ecosystem and providing much of the energy to the earth. In the model plant Arabidopsis, stomatal development and patterning are regulated by a linear signal pathway, including peptide ligands, receptors, MAPK cascade, and a series of transcription factors. The MAPK cascade receives upstream signals from the cell surface to transduce to intracellular factors, thus plays a central role in the regulation of stomatal development (Fig. 1). Therefore, it is critical to investigate the signaling events upstream and downstream of the MAPK cascade to better understand the stomatal production and patterning.

In the first chapter of my thesis, I determine the function of the PP2A phosphatase complex in promoting stomatal development by stabilizing the key transcription factor SPCH, thus reveal the missing phosphatase that combats with MPK3/6 and other known kinases to balance the phosphorylation of SPCH (Fig. 1).

*Outstanding questions related to PP2A function and SPCH degradation:*

- How this regulation is integrated in the environmental context and what signals trigger this regulation will be interesting to investigate in the future.

- The B subunits are commonly accepted to confer functional specificity to the PP2A complexes, thus it is important to identify which B subunits may participate in stomatal regulation.
• In addition, it is critical to identify what upstream signals (hormonal or environmental) that controls proper function of PP2A complex in stomatal lineages.

In the second chapter, my work together the collaboration with Dr. Kezhen Yang describes that the polarity D6PK proteins associate with the BASL-YDA polarity complex at the cell cortex and possibly regulate the complex activity, thus providing insights of the polarity regulation in stomatal development and patterning (Fig. 1).

Further investigation is required to better understand how D6PK family may regulate BASL-YDA complex and other polarity proteins.

**Outstanding questions related to D6PK function:**

• Our data suggest D6PK participates in the BASL-YDA polarity complex to regulate stomatal patterning, but this model may not explain the D6PK overexpression-generated phenotypes. The overexpression of D6PK may not disrupt BASL polarity but it leads to stomatal patterning defects, suggesting that some unknown targets, especially the ones at the plasma membrane, are possibly regulated by D6PK. Besides the BASL-centered intrinsic polarity complex, an extrinsic genetic pathway that ensures stomatal patterning is the peptide ligand-receptor (EPF1-ERL1-TMM) pathway. Whether and how these signaling components can be targeted and regulated by D6PK are significant questions to be addressed.

• Also, D6PK is a well-known kinase that phosphorylates and regulates auxin transporter PIN proteins in many tissue-specific responses. Previous studies
showed high-order mutants of PIN proteins exhibit stomatal patterning defects, could this be the consequence of D6PK phosphorylation? Future studies are anticipated to address the functional connection between D6PK-PIN in the regulation of stomatal development and patterning.

- Recent studies identified a D6PK-like protein family, the PAX proteins, in the regulation of the polarity of BRX, but auxin negatively regulates the plasma membrane association of BRX and promotes PAX activity in the roots. Considering that D6PK and PAX members are close homologues and the inter-dependency of BASL and BRX in the polarity formation, it would be interesting to test if D6PK regulates BRX protein, and if there is any interaction between the PAX-BRX complex and the BASL-YDA-D6PK polarity complex in stomatal development and patterning.

In the last chapter, through a collaborative effort with Dr. Xueyi Xue and Dr. Xiaoyu Guo, we characterize a new family of MAPK substrate, the MASS proteins in stomatal development. Our data suggest MAPK-mediated phosphorylation regulates the MASS subcellular localization, and in turn the MASS proteins interact with YDA and confer a positive regulation to stomatal production.

**Outstanding questions related to MASS function and MAPK signaling:**

- The functional connection between MASS and the MAPK cascade signaling may represent a negative feedback regulation (Fig. 1). Future study should target on the molecular mechanism for MASS and its partners to promote stomatal development at the plasma membrane. Our data suggest MASS
proteins interact with the MAPKKK YDA at the cell cortex, but how MASS proteins mechanistically suppress YDA functions requires more in-depth investigation.

- Besides, the MASS overexpression generated stomatal cluster phenotype suggests MASS proteins participate in stomatal patterning regulations. One genetic pathway that regulates stomatal patterning is the peptide ligand-receptor signaling (EPF1-ERL1-TMM-SERK). It would be important to test if plasma membrane-localized MASS proteins interact and affect the function of these signaling components.

**In conclusion,** in this thesis, my studies reveal the connected functions of several newly identified stomatal regulators that are integrated into the hub signaling module, the MAPK cascade, at the genetic and molecular level. The expanded list of stomatal signaling molecules and regulators from this study will further advance our mechanistic understanding on the molecular mechanisms underlying cell division, cell fate specification, cell polarity, cell-cell communication and signaling events in responding to environmental changes, etc., all of which are integrated into properly numbered and patterned stomatal formation in higher plants.
Fig. 1. Current understanding of stomatal development and patterning in Arabidopsis

The diagram illustrates the integration of my studies in this thesis into the current genetic pathway that regulate stomatal development and patterning. In the first chapter, I describe the positive role of PP2A phosphatase in stomatal formation by stabilizing the key transcription factor SPCH. In the second chapter, I characterize the D6PK protein family participates in the BASL-YDA polarity complex and controls stomatal development and patterning. In the last chapter, we propose that the newly identified MAPK substrate MASS family may form a negative feedback loop with the YDA MAPK cascade and positively regulate stomatal production.