

**ELUCIDATING THE MECHANISM OF ER-FAMILY RECEPTORS IN REGULATION
OF STOMATAL DEVELOPMENT AND SHOOT APICAL MERISTEM**

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ABSTRACT

ERECTA-family receptors (ERfs) that contains ERECTA (ER), ERECTA LIKE 1 (ERL1), and ERECTA LIKE 2 (ERL2) are important for diverse developmental processes in *Arabidopsis*, such as organ elongation, stomatal formation, reproductive development, shoot apical meristem (SAM) maintenance, and leaf morphology. The activity of ERfs is regulated by protein ligands that belong to the EPIDERMAL PATTERNING FATOR/ EPF LIKE (EPF/ EPFL) family. There are 11 EPF/ EPFL ligands in *Arabidopsis*. Over the past years, we have gained better understanding of how ERfs regulate *Arabidopsis* development, and which EPF/ EPFL ligands are responsible for triggering ERf signal in which developmental process. However, on the protein level we did not know how ERfs interact with EPF/ EPFL ligands or how the receptors identify different ligands. The SAM controls the generation of aboveground organs throughout plant life. The *er erl1 erl2* mutant has an enlarged SAM and exhibits reduced leaf initiation. How the SAM is regulated by ERfs remained unclear. Work presented in this dissertation advances our knowledge in:

- 1) The mechanism of interactions between ERfs and EPF/ EPFL ligands. My work showed that the function of EPF1 was reduced when S3, G13, or P50 was mutated, and that TMM lost its function when E379 was mutated. My work also provided evidence showing that EPFL9 suppressed the negative regulation of EPF1 on stomatal formation by competing with EPF1 for receptor binding.
- 2) The contribution of ER-family receptors in the SAM maintenance. Our genetic analyses revealed that ER-family receptors and CLV3 synergistically suppress SAM expansion, and *WUS* is epistatic to *ER*-family genes. The expression of *WUS* and *CLV3* was up-regulated in the *er erl1 erl2* mutant. Since EPF/ EPFL ligands are expressed at the SAM boundary, it is proposed that ERf signal laterally restricts the expression of *WUS* and *CLV3* to the central region of the SAM.

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CHAPTER 1: INTRODUCTION

ERECTA family receptors

Arabidopsis thaliana Landsberg *erecta* (*Ler*) is a widely used model system in plant research. *Ler* was isolated from X-ray mutagenized Landsberg seeds by György P. Rédei in 1957 (Rédei 1962). It harbors the *erecta* (*er*) mutation (Rédei 1992). The *ERECTA* (*ER*) gene encodes a leucine-rich repeat receptor like kinase (LRR-RLK) (Torii et al. 1996). The ER protein contains an extracellular domain consisting of 20 tandem copies of a 24-amino acid leucine-rich repeat, an one-cross transmembrane region, and an intracellular serine/threonine kinase domain (Figure 1.1). The intracellular kinase domain of ER is flanked by a juxtamembrane domain and a C-terminus tail. The juxtamembrane domain is important for ER's function, but the C-terminus tail is not (Kosentka et al. 2017). ER containing truncated juxtamembrane domain was unable to fully rescue the inflorescence phenotype of the *er* null mutant. However, deletion of the C-terminus tail did not influence ER's function.

In *Arabidopsis* there are two homologs of ER, which are ERECTA LIKE 1 (ERL1) and ERECTA LIKE 2 (ERL2) (Shpak et al. 2004). ERL1 and ERL2 share 62% sequence identity and 73% similarity to ER. The LRR region and the kinase domain of ER-family receptors (ERfs) exhibit the highest sequence conservation, while the N-terminal signal sequence and the C-terminal tail are poorly conserved. The expression patterns of *ER*, *ELR1*, and *ERL2* are largely overlapping (Shpak et al. 2004). All three genes are expressed in young developing organs, such as young rosettes, flower buds, and siliques. However, *ER* is also relatively highly expressed in stem and mature leaves, indicating ER has unique roles in *Arabidopsis* development. Nevertheless, this uniqueness of ER is likely determined by its

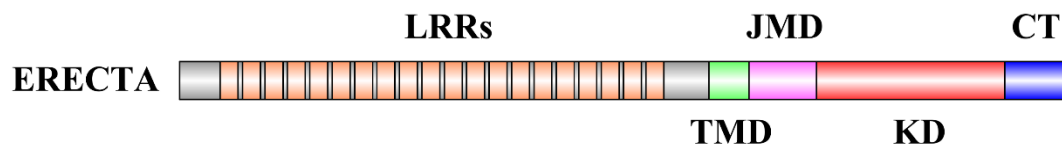


Figure 1.1 Schematic diagram of ERECTA. (LRRs) leucine-rich repeats, in orange; (TMD) transmembrane domain, in green; (JMD) juxtamembrane domain, in pink; (KD) kinase domain, in red; (CT) C-terminus tail, in blue.

differential expression, rather than ER functions differently from ERL1/ ERL2. When *ERL1* and *ERL2* were expressed under the control of *ER*'s promoter and terminator, *ERL1* and *ERL2* could rescue the *er* null mutant phenotype (Shpak et al. 2004). This suggests that ER, ERL1, and ERL2 are able to bind the same ligands and activate/ inactivate the same downstream signaling components.

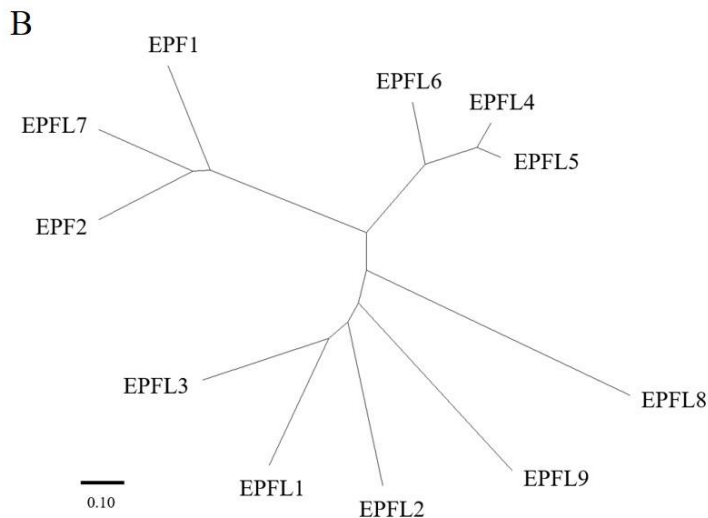
ERfs regulate diverse aspects of plant development such as stomatal patterning (Shpak et al. 2005), organ elongation (Shpak et al. 2004), anther and ovule development (Pillitteri et al. 2007a; Hord et al. 2008), leaf tooth growth (Tameshige et al. 2016) and the maintenance of the shoot apical meristem and phyllotaxis (Uchida et al. 2013; Chen et al. 2013). ERfs also modulate plant responses to pathogens (Llorente et al. 2005; Sánchez-Rodríguez et al. 2009; Jordá et al. 2016) and abiotic stresses (Van Zanten et al. 2010; Patel et al. 2013).

EPF/ EPFL family ligands

The activity of ERfs is regulated by ligands that belong to the EPIDERMAL PATTERNING FACTOR/ EPF LIKE (EPF/ EPFL) family. There are 11 EPF/ EPFL ligands in *Arabidopsis* (Hara et al. 2009). EPF1, EPF2 and EPFL9 coordinately regulate stomatal patterning (Lee et al. 2015; Lin et al. 2017). EPFL4 and EPFL6 promote organ elongation and are also able to repress stomatal formation (Abrash and Bergmann 2010; Abrash et al. 2011). EPFL2 is required for the outgrowth of leaf tooth on leaf margin (Tameshige et al. 2016). EPFL1, EPFL2, EPFL4, and EPFL6 redundantly restrict the shoot apical meristem size and promote leaf initiation (Kosentka et al. 2019). The functions of EPFL3, EPFL5, EPFL7, and EPFL8 are unclear.

NMR and crystal structures revealed that EPF/ EPFL ligands are composed of an antiparallel β -sheet, a hairpin loop that connects the two β -strands, and N- and C- terminal coils (Ohki et al. 2011; Lin et al. 2017). EPF/ EPFL ligands have six conserved cysteines residues (Figure 1.2), which form intramolecular disulfate bonds and are important for structural integrity. The β -sheet and the N-terminal coil cover the regions showing relatively high conservation among the 11 ligands. In contrast, the hairpin loop and the C-terminal coil display a large divergence.

EPFL9	IGSTAPTCTYNECRGCRYKRAEQVPVEGNDP-----INSAYHYRCVCHR-----	45
EPFL2	IGSRPPRCERVRRCSCG-HCEAIQVPTNPQTKLHSPLTSSSSSSSETHLDYTRGDSTNYKPMKSWCKCKGNSIYNP----	76
EPFL1	LGSTPPSCHN-RCNCH-PCMAIQVPTLPTRSRFRTRVNPFSGGFVRPPSSSLTTVLQDQSYNYKPMGWKCHCNHGFIYNP----	75
EPFL3	MGSEPPCEK-KCYGCE-PCEAIQEPTTIS-----IPHLSPHYANYQFEGWRCHCPP-----	51
EPFL8	MGSEPPVCAT-KCRNCK-PCLPYLFDIRGAH-----DDDDSEPIYPVKWTCRCRDVFEF-----	54
EPFL6	LGSSPPRCSS-KCGRCT-PCPKVHVVPVPGT-----P---VTAEYYPEAWRCKCKGNKLYMP-----	51
EPFL4	FGSSPPTCRS-KCGKCQ-PCPKVHVPIQPL-----S---MPLEYYPEAWRCKCKGNKLFMP-----	51
EPFL5	PGSVPPMCLRC-KCGKCE-PCKAVHVPIQPL-----I---MPLEYYPEAWRCKCKGNKLFMP-----	51
EPFL1	AGSRLPDCSH-ACGSCS-PCRLVMVSFVCA-----V---EEAETCPMAYKCMCNKSYVPV-----	52
EPFL2	TGSSLPDCSY-ACGACS-PCKRVMISFECSV-----AESCSVIYRCTCRGRYHYVPSRA-----	52
EPFL7	SGSSIPDCSN-ACGPCK-PCKLVISSSTCSA-----SEAQLPVYKCLCKGKYHYVPSLT-----	52
	** * *	



4

Protein alignment reveals that EPF/ EPFL ligands have a conserved “GS” motif in their N-terminal coils, which is consisted of a glycine residue and a serine residue (Figure 1.2). This “GS” motif is crucial for the binding of EPF/ EPFL ligands to ERfs. Crystal structures showed that the “GS” motifs of EPF1, EPF2, and EPFL4 formed a set of conservatively polar interactions with ERL1 and ERL2 (Lin et al. 2017). When the serine residue of the EPF1 “GS” motif was mutated to an arginine residue, the affinity of EPF1 to ERL1 was reduced and the mutated EPF1 was unable to efficiently suppress stomatal formation (Lin et al. 2017).

The hairpin loop of EPF/ EPFL ligand determines ligand’s function. EPF2 suppresses stomatal formation (Hunt and Gray 2009), whereas EPFL9 has stomata-inducing activity (Sugano et al. 2010). However, the chimeric ligand (EPF2’-St) consisting of the EPF2 scaffold and the EPFL9 hairpin loop was unable to inhibit stomatal formation. Instead, *Arabidopsis* seedlings treated with this ligand were found to have increased stomatal density of cotyledons. Unlike EPF2’-St, the reciprocal chimera (St’-EPF2), which contains the EPF2 hairpin loop and the EPFL9 scaffold, reduced stomatal density of cotyledons (Ohki et al. 2011).

It is proposed that the hairpin loop of the EPF/ EPFL ligand might interact with other plasma membrane proteins and mediate the formation of different protein complexes between those proteins and ERfs in order for ERfs to transduce diverse signals. PHLOEM INTERCALATED WITH XYLEM (PXY) is an LRR-RLK (Fisher and Turner 2007). Its activity is regulated by a small peptide named TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) (Hirakawa et al. 2008). The binding of TDIF to PXY recruits SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 2 (SERK2), which acts as a coreceptor with PXY for regulating vascular development (Zhang et al. 2016). The structural superposition of EPF1-ERL1^{LRR}-TMM^{LRR} over TDIF-PXY^{LRR}-SERK2^{LRR} showed that EPF1 was oriented in a way that its hairpin loop might be able to interact with SERK2 (Lin et al. 2017). The *serk1-1 serk2-1 serk3-5* mutant phenocopied the *erl1 erl2* mutant in the aspects of suppressed stomatal formation and petiole/pedicle elongation (Meng et al. 2015). Coimmunoprecipitation assays indicated that ER and ERL1

coimmunoprecipitated with SERK1, SERK2, SERK3, and SERK4. The interactions between ERfs and SERKs ligand dependent. The application of EPF1 or EPF2 enhanced the association of ERfs with SERKs (Meng et al. 2015). These suggest that SERKs might function as signaling components of the ERf pathway.

ERfs and EPF/ EPFLs in regulation of stomatal development

Stomata are small pores in the epidermis of the aboveground organs of land plants. They control gas exchange and transpiration. Stomata are generated from protodermal cells, which undergo a series of cell divisions and cell-fate transitions (Figure 1.3) (Shpak 2013). Early in stomatal development, a subset of protodermal cells, called meristemoid mother cells (MMC), undergo asymmetrical division and produce two daughter cells that are different in size. The large daughter cell is called stomatal lineage ground cell (SLGC). The small daughter cell is called meristemoid. The meristemoid continues several rounds of asymmetric proliferation, resulting in a centrally localized meristemoid surrounded by SLGCs. The meristemoid eventually differentiate into a guard mother cell (GMC), which then divide symmetrically to give rise to two guard cells that compose a stoma. The SLGCs can either adopt the fate of pavement cell or undergo asymmetrical divisions to generate satellite meristemoids and stomata.

ERfs are crucial for stomatal formation and patterning. Complete loss of all three *ER*-family genes leads to a significant increase in stomatal generation. In *Arabidopsis* stomata are normally separated by at least one nonstomatal epidermal cell. Whereas the *er erl1 erl2* mutant exhibits a stomata clustering phenotype (Shpak et al. 2005). ER-family receptors redundantly regulate stomatal development while also show functional divergence. Genetic analyses revealed that ER primarily prevents MMC transition. In contrast, ERL1 acts as the major receptor that suppresses GMC transition (Shpak et al. 2005). EPF1 and EPF2 are the two ligands that activate ER-family receptors in regulation of stomatal development. Overexpression of *EPF1* or *EPF2* results in no stomatal formation as stomatal lineage cells exclusively choose to become pavement cells (Hara et al. 2007; Hunt and Gray 2009). EPF1 is made by meristemoids, GMCs and early guard cells. It diffuses to neighbor cells and

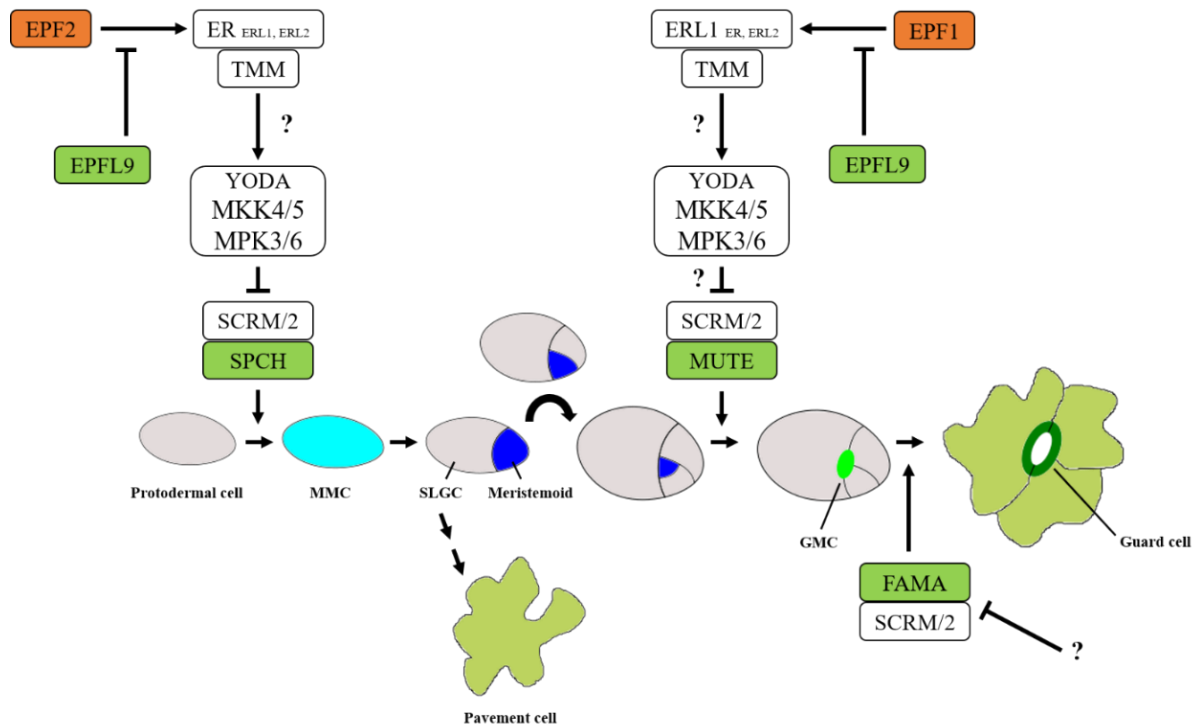


Figure 1.3 Model for the role of ERF signaling in stomatal development. The first step of stomatal development is the transition of protodermal cell to meristemoid mother cell (MMC). This step is negatively regulated by ERF signaling triggered by EPF2. ER is the major receptor involved in this step. ERF signal is transduced by a MAPK cascade that consists of YODA (MAPKKK), MKK4/5 (MAPKKs), and MPK3/6 (MAPKs). The MAPK cascade inactivates the transcription factor SPCH, which promotes the MMC differentiation. MMC undergoes an asymmetrical division and produces a smaller meristemoid and a larger stomatal lineage ground cell (SLGC). The meristemoid continues several rounds of asymmetric proliferation and then differentiates into guard mother cell (GMC). This process is suppressed by EPF1 induced ERF signal. ERL1 is the primary receptor of EPF1. ERF signal via the same MAPK cascade inactivates MUTE, the transcription factor that promotes the GMC differentiation. It is not clear whether MUTE is directly inactivated by MPK3/6. Another transcription factor FAMA controls the transition of GMC to guard cells, but it is unknown how FAMA is regulated. SPCH, MUTE, and FAMA form heterodimers with SCRM/2 in order to function. The binding of EPF1 and EPF2 to ER-family receptors requires coreceptor TMM. EPFL9 positively regulate stomatal formation by acting as an antagonist of EPF1 and EPF2 to prevent ER-family receptors from being activated. How is ERF signal transduced from the receptor to YODA has remained unknown.

prevent them from turning into stomata (Hara et al. 2007). EPF2 is made by MMCs and early meristemoids. It prevents neighbor cells from entering a stomatal lineage fate (Hunt and Gray 2009). EPFL9, which promotes stomatal formation, competes with EPF1 and EPF2 to bind ER-family receptors, but its binding does not activate downstream signaling events (Lee et al. 2015; Lin et al. 2017). EPFL9 likely functions as an antagonist of EPF1 and EPF2 to occupy the ligand binding site in ER-family receptors and to prevent signal transduction.

The binding of EPF1 and EPF2 to ER-family receptors requires coreceptor TOO MANY MOUTHS (TMM). TMM is a plasma membrane-located leucine-rich repeat protein, which lacks intracellular domain (Nadeau and Sack 2002). The *tmm* mutant displays increased stomatal formation and stomata clustering on the epidermis of cotyledons and leaves, which phenotype is indistinguishable from the *erl1 erl2* mutant (Yang and Sack 1995; Shpak et al. 2005). Overexpression of *EPF1* or *EPF2* in the *tmm* mutant background failed to reduce stomatal density (Hara et al. 2007; Hara et al. 2009). TMM interacts with ER-family receptors in a ligand independent manner. The formed protein complexes create a groove, in which EPF1 or EPF2 insert (Lee et al. 2012; Lin et al. 2017). The comparison of the *tmm* mutant and the *erl1 erl2* mutant raises an intriguing question. If TMM and ER-family receptors function cooperatively in regulation of stomatal development, why does the *tmm* mutant produce no stoma in hypocotyl or stem, while the *erl1 erl2* mutant forms more stomata and stomatal clusters? EPFL4 and EPFL6, two ligands of ER-family receptors that promote stem elongation, are able to repress stomatal formation (Abrash et al. 2011). However, unlike EPF1 or EPF2, EPFL4 and EPFL6 reduce stomatal density when functional TMM is not present. Thus, an exciting scenario is that TMM determines the ligand specificity of ER-family receptors. This idea is supported by our recent work. Protein pull-down assays showed that EPFL4 and EPFL6 interacted with ER-family receptors in the absence of TMM, whereas neither EPF1 nor EPF2 did. Isothermal titration calorimetry data showed that the presence of TMM increased the binding affinity of EPF1 to ERL1 from no detectable binding to 1.2 μM (dissociation constant), but decreased the affinity of EPFL4 from 0.14 μM to 1.3 μM (Lin et al. 2017). It is proposed that in hypocotyls and stems of the *tmm* mutant, EPFL4 and EPFL6, which are expressed in endodermis, diffuse to epidermis, where they replace EPF1 and EPF2 to inhibit stomatal formation. Because EPFL4 and EPFL6 interact with ER-family receptors

very efficiently, stomatal formation is dominated by negative regulation, resulting in no stoma forming. As in hypocotyls and stems of the *er erl1 erl2* mutant, because no ligand can trigger ERf signal, the restriction on stomatal formation is removed, causing more stomata and stomatal clusters to form.

ERf signal in stomatal development is transduced by a MAPK cascade that consists of YODA (MAPKKK), MKK4/5 (MAPKKs), and MPK3/6 (MAPKs). The same MAPK cascade seems to be also involved in regulation of organ elongation, anther and ovule development, and shoot apical meristem maintenance (Pillitteri et al. 2007a; Hord et al. 2008; Meng et al. 2012; Chen et al. 2013). Loss of function of these kinases lead to massive stomatal formation. Conversely, expression of constitutively active kinases eliminates stomata (Bergmann et al. 2004; Wang et al. 2007). Mutants of *yoda*, *mkk4/5*, and *mpk3/6* exhibit much severe stomatal development defects than mutants of *er erl1 erl2*, *tmm*, or *epf1 epf2*. The *mpk3 mpk6* double mutant and the *MKK4 MKK5* RNAi silencing plant have the entire epidermis of cotyledons be composed of stomata, and their growth is arrested at the cotyledon stage (Wang et al. 2007). This suggests that other signals in addition to ERf signal also regulate stomatal development via this MAPK cascade. How is ERf signal transduced from the receptor to YODA has remained unknown. Cell fate transitions of stomatal lineage cells are controlled by three basic-helix-loop-helix (bHLH) transcription factors: SPEECHLESS (SPCH), MUTE and FAMA (Ohashi-Ito and Bergmann 2006; MacAlister et al. 2007; Pillitteri et al. 2007). SPCH promotes the transition of MMC to meristemoid. MUTE promotes the transition of meristemoid to GMC. FAMA promotes GMC to undergo a symmetrical division and become guard cells. These three transcription factors regulate stomatal development in forms of heterodimers with another two bHLH transcription factors SCREAM (SCRM) and SCRM2 (Kanaoka et al. 2008). MPK3 and MPK6 can phosphorylate SPCH in vitro, while elimination of those phosphorylation sites creates hyperactive SPCH variants (Lampard et al. 2008). Genetic analysis showed that *MUTE* is epistatic to *ER*-family genes (Pillitteri et al. 2007), but whether MUTE is directly inactivated by MPK3/6 is not clear. The signal that regulates FAMA is still undisclosed.

ERfs and EPF/ EPFLs in regulation of the shoot apical meristem

The shoot apical meristem (SAM) is an indeterminate structure at the top tip of a plant, which functions as a reservoir of stem cells that is necessary for generating all post-embryonic aerial organs (Figure 1.4). In the dome-shaped SAM, stem cells are located in the central zone (CZ). Stem cells slowly proliferate, and their progenies are displaced laterally into the peripheral zone (PZ) or basally into the rib zone (RZ). Cells in the PZ and the RZ will ultimately differentiate to form leaves, flowers, and stems. The SAM consists of three clonally distinct cell layers (L1–L3). Cells in the L1 and L2 undergo anticlinal cell division and give rise to epidermis and ground tissues respectively. Cells in the L3 divide in all directions and produce vascular tissues.

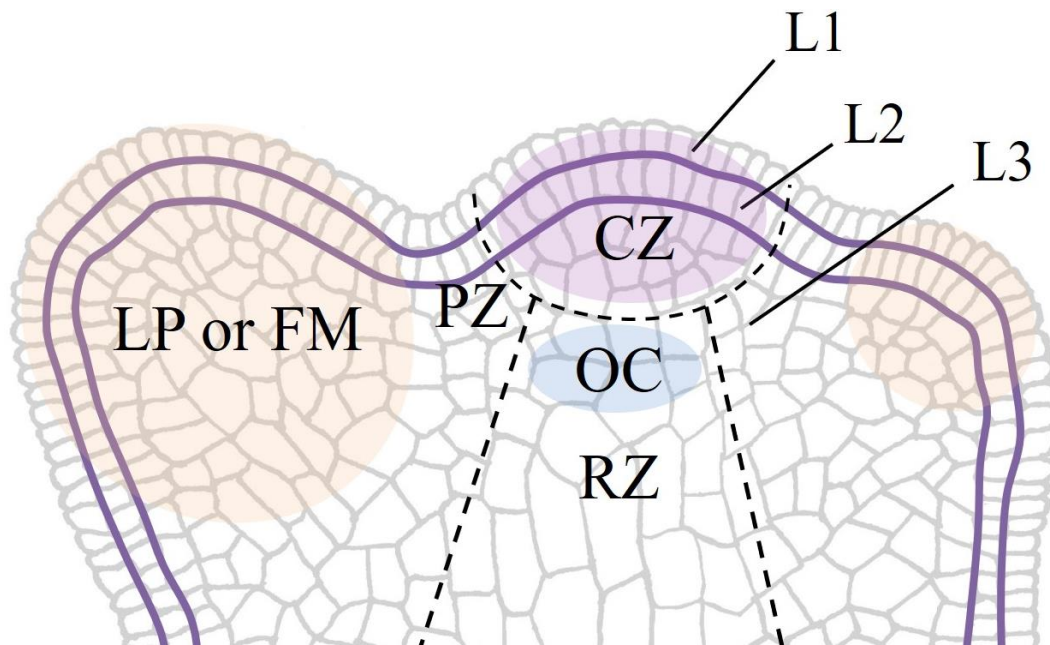


Figure 1.4 Schematic representation of the SAM. The dome-shaped SAM is divided into three zones: central zone (CZ), the peripheral zone (PZ) and the rib zone (RZ), and three layers: L1, L2 and L3. Stem cells locate in the CZ. Daughter cells generated by stem cell proliferation enter the PZ and the RZ. Cells in the PZ divide and eventually differentiate to form leaf primordium (LP) or flower meristem (FM) that will produce flower organs. Cells in the RZ contribute to stem growth. Underneath the CZ there is a small group of cells that consist of the organization center (OC). The OC is crucial for the maintenance of stem cell homeostasis in the CZ. (Purple region) CZ; (yellow region) LP or FM; (blue region) OC.

Despite the fact that the SAM is constantly creating new organs, the number of stem cells in the CZ is stable throughout plant development. Stem cell homeostasis is controlled by an intercellular communication between the CZ and the organization center (OC) that is located underneath the CZ (Figure 1.5). Cells in the OC express a homeodomain transcription factor called WUSCHEL (*WUS*) (Mayer et al. 1998). *WUS* proteins move from the OC to the CZ through plasmodesmata (Yadav et al. 2011; Daum et al. 2014), and in the CZ *WUS* proteins promote stem cell proliferation. Stem cells are absent in the shoot apex of the *wus* mutant, resulting in the lack of the dome-shaped SAM (Laux et al. 1996). Stem cells express and secrete a small peptide named CLAVATA 3 (*CLV3*). *CLV3* is recognized by plasma membrane localized receptors, such as CLAVATA 1 (*CLV1*), *CLV2*, CORYNE (*CRN*), RECEPTOR-LIKE PROTEIN KINASE 2 (*RPK2*), and BARELY ANY MERISTEM (*BAM*) (Kayes and Clark 1998; Fletcher et al. 1999; DeYoung et al. 2006; Müller et al. 2008; Kinoshita et al. 2010). *CLV3* signal represses *WUS* expression (Brand et al. 2000). In *clv* mutants *WUS* is over-expressed, resulting in an enlarged SAM with expanded CZ and OC.

ER, *ERL1*, and *ERL2* are expressed in the SAM (Yokoyama et al 1998; Uchida et al. 2013). They redundantly regulate SAM size and leaf initiation. The *er erl1 erl2* mutant has a

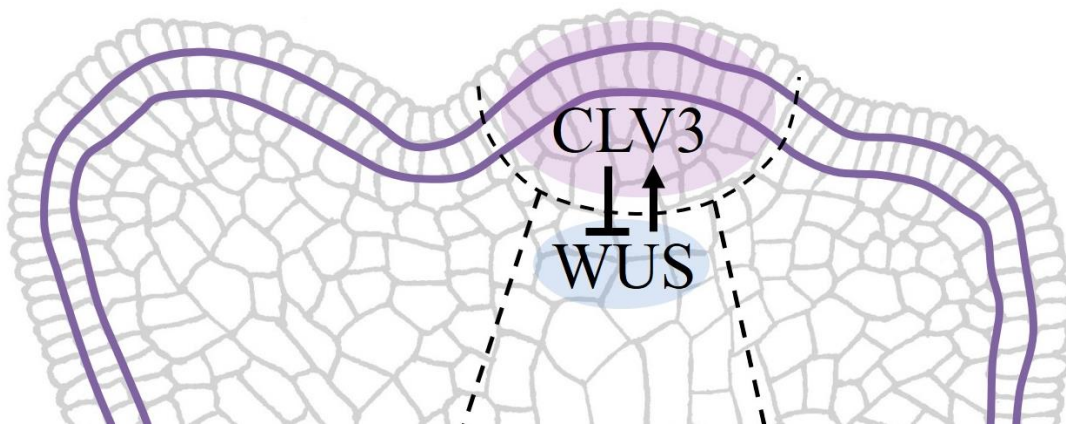


Figure 1.5 The SAM is regulated by the *WUS*-*CLV3* negative feedback loop. *WUS* proteins are made by cells in the organization center (OC, blue region). *WUS* proteins move from the OC to the central zone (CZ, purple region). In the CZ, *WUS* promote stem cell proliferation. Stem cells secrete *CLV3*, a small peptide, which diffuses downwards and binds its receptors to trigger signals to repress *WUS* expression in the OC.

flat and broadened SAM compared with the wt SAM, while neither the *erf* single mutants nor the double mutants exhibit apparent abnormality in SAM morphology (Uchida et al. 2013; Chen et al. 2013). Leaf primordia are initiated at a significantly decreased rate with disrupted phyllotaxis in the *er erf1 erf2* mutant (Chen et al. 2013). The defects of the *er erf1 erf2* mutant in leaf development does not appear to be correlated with the dysregulation of SAM size. Unlike the *er erf1 erf2* mutant, the *clv3* mutant, which also has an enlarged SAM, initiates leaf primordia in an increased rate (Vernoux et al 2000). Leaf initiation relies on the establishment of auxin maxima at the position of presumptive leaf primordia within the SAM (Reinhardt et al. 2000). Analysis of the *DR5rev::GFP* reporter revealed an even distribution of GFP fluorescence in the L1 of the *er erf1 erf2* SAM (Chen et al. 2013), suggesting leaf initiation defects of the *er erf1 erf2* mutant was due to an abnormal auxin distribution.

ERfs might restrict SAM size via repressing *WUS* expression. *WUS* expression was up-regulated in the *er erf1 erf2* mutant. In addition, RNA in-situ hybridization revealed that the *er erf1 erf2* mutant displayed an expanded *WUS* expressing region (Uchida et al. 2013; Chen et al. 2013). In a recent publication, Kimura and colleagues indicated that ERfs regulate SAM size independently from *WUS* (Kimura et al. 2018). The authors found stem cells that were missing in the *wus* single mutant were present in the *wus erf erf1 erf2* quadruple mutant. However, a main concern about their finding is that stem cells observed in the quadruple mutant might not be due to loss of function of ER-family genes. Although the *wus* mutant lacks a SAM, it still produces leaves and flowers. This is because the *wus* mutant is able to develop secondary shoot meristems across its shoot apex (Laux et al. 1996), which suggests cells in the *wus* shoot apex can somehow acquire stem cell identity.

CLV3 and cytokinin (CK) signals function as the positional cues for limiting *WUS* expression to the OC in a longitudinal manor (Chickarmane et al. 2012). *CLV3* is expressed in the CZ, from which *CLV3* ligands diffuse downwards and repress *WUS* expression along the way of diffusion. The CK biosynthetic gene, *LONELY GUY (LOG)*, is expressed in the epidermis of the SAM. CK produced in the epidermal layer also forms an apical-basal diffusion gradient within the SAM. CK signal promotes *WUS* expression (Gordon et al. 2009), but only in the OC, where the CK level is relatively low (Chickarmane et al. 2012). In

the CZ the high level of CK activates type-A *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*) that in turn down-regulates CK signal. Whereas in the OC type-A *ARRs* are repressed by high level of *WUS*, resulting in a relatively high level of CK signal in the OC and a positive feedback loop between CK signal and *WUS* expression.

WUS activates *CLV3* by directly binds to *CLV3* promoter (Yadav et al. 2011). However, *CLV3* is not expressed in the OC. HAIRY MERISTEMS (*HAMs*) are GRAS-domain transcription factors that act as interacting partners of *WUS* in regulation of stem cell homeostasis (Zhou et al. 2015). Recent research indicated that *HAMs* longitudinally shaped the pattern of *CLV3* expression (Zhou et al. 2018). The expression of *HAM* genes formed a basal-apical gradient in the SAM, with the highest expression in the OC while no expression in the L1 and L2, which was complementary to the distribution of *CLV3* transcripts. Mathematic modeling and RNA in-situ hybridization showed that knocking out *HAM* genes led to a shift of *CLV3* expression from the CZ to the OC. It is proposed that the interaction of *WUS* and *HAM* in the OC represses *CLV3* expression, but in the CZ, where *WUS* is unlikely to interact with *HAM*, *WUS* is able to turn on *CLV3* transcription.

A question remained is that whether the expression of *WUS* and *CLV3* is also laterally regulated. If the answer is yes, what are the signals? The expression regions of *WUS* and *CLV3* were broadened in the *er erl1 erl2* SAM (Uchida et al. 2013; Kimura et al. 2018), suggesting that ERf signal might contribute to the lateral restriction on *WUS* and *CLV3*. *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* function redundantly in regulation of SAM size (Kosentka et al. 2019). The *epfl1 epfl2 epfl4 epfl6* mutant has an enlarged SAM that is comparable to the *er erl1 erl2* SAM. *EPFL1*, *EPFL2*, and *EPFL4* are expressed in the boundary region of the SAM. These EPFL ligands might provide the positional cues for laterally limiting *WUS* and *CLV3* expression.

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CHAPTER 2: A RECEPTOR-LIKE PROTEIN ACTS AS A SPECIFICITY SWITCH FOR THE REGULATION OF STOMATAL DEVELOPMENT

Work presented in this chapter is included in the following publication:

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Abstract

Stomata control water and carbon dioxide movement between plants and the environment. These microscopic pores are important for plant survival and the global water and carbon cycles. Stomatal development is regulated by ERECTA-family receptors (ERfs), EPIDERMAL PATTERNING FACTOR/ EPF LIKE (EPFL) family ligands, and coreceptor TOO MANY MOUTHS (TMM). Our work elucidated the mechanism of interactions between ERfs, EPFLs, and TMM. Based on crystal structures of ERf-TMM-EPFL protein complexes, we found residues involved in the receptor-ligand interaction. Here I tested whether those residues were functionally important in vivo. My results showed that the function of EPF1 was reduced when S3, G13, or P50 were mutated, and that TMM lost its function when E379 was mutated. Here I also report that EPFL9 prevents EPF1 from repressing stomatal formation. Taken together, my data support our protein crystal structures and reveal residues that are important for the function of EPF1 and TMM.

Introduction

Stomata are microscopic pores on the epidermis of land plants, which control diffusive paths of water and carbon dioxide between plants and the atmosphere, thus optimizing water use efficiency and regulating photosynthesis. Proper stomatal formation is crucial for plants to survive and plays an important role in global water and carbon cycles (Hetherington and Woodward 2003). Stomata are formed from protodermal cells. In the beginning of stomatal development, protodermal cells adopt stomatal lineage cell fate and become meristemoid mother cells (MMCs). An MMC undergoes an asymmetrical division and give rise to a big daughter cell called stomatal lineage ground cell (SLGC) and a small daughter cell called meristemoid. The meristemoid continues several rounds of asymmetric cell divisions and eventually differentiate into a guard mother cell (GMC). The GMC symmetrically divide and produce two guard cells, which form a stoma.

Stomatal development is regulated by ERECTA-family receptors (ERfs), which are plasma membrane localized leucine-rich repeat receptor like kinases (LRR-RLKs) (Shpak et al. 2005). *Arabidopsis* has three ERfs: ERECTA (ER), ERECTA LIKE 1 (ERL1) and ERECTA LIKE 2 (ERL2). A mitogen-activated protein (MAP) kinase signaling pathway functioning downstream of ERfs contains MAP kinase kinase kinase (MAPKKK) YODA, MAP kinase kinase (MAPKK) 4/5 and MAP kinase (MAPK) 3/6 (Bergmann et al. 2004; Wang et al. 2007; Lampard et al. 2009). This MAPK cascade phosphorylates transcription factors SPEECHLESS (SPCH) and MUTE, which promote stomatal development (MacAlister et al. 2007; Pillitteri et al. 2007). After being phosphorylated, SPCH and MUTE are degraded.

In epidermis, ERf signaling is triggered by protein ligands that belong to the EPIDERMAL PATTERNING FACTOR/ EPF LIKE (EPFL) ligand family. There are 11 EPF/ EPFL ligands in *Arabidopsis*. These ligands are composed of an antiparallel β -sheet, a hairpin loop located between the two β -strands, and two terminal loops (Ohki et al. 2011; Lin et al. 2017). EPF/ EPFL ligands have 6 conserved cysteine residues that form intramolecular disulfide bonds to stabilize ligand's structure (Ohki et al. 2011). They also have a conserved

“GS” motif at their N-terminal ends, which consists of a glycine and a serine. This “GS” motif mediates the interactions between EPF/ EPFL ligands and ERfs (Lin et al. 2017). EPF1 and EPF2 are the two ligands that suppress stomatal formation. EPF2 is made by MMCs and early meristemoids. It diffuses to neighboring cells and prevents them from entering stomatal lineage cell fate (Hara et al. 2009; Hunt and Gray 2009). EPF1 is made in the late stages of stomatal development by late meristemoids, GMCs and early guard cells. It inhibits nearby cells from differentiating and forming guard cells (Hara et al. 2007). EPFL9 is produced by mesophyll cells (Kondo et al. 2009). It diffuses to the epidermis, where it positively regulates stomatal formation (Sugano et al. 2010). In the epidermis, EPFL9 competes with EPF2 for binding to ERfs, but its binding cannot activate the receptors (Lee et al. 2015). In vitro protein pull-down assay revealed that EPFL9 competed with EPF1 for binding with ERfs (Lin et al. 2017). When the concentration of EPFL9 was increased, less ERL1 proteins was pulled down by EPF1 (Lin et al. 2017). Here I show this competition also happened in vivo. *Arabidopsis* seedlings treated with EPF1 had stomatal formation repressed, whereas seedlings cotreated with EPF1 and EPFL9 produced more stomata and stomatal clusters.

TOO MANY MOUTHS (TMM) is a plasma membrane localized receptor like protein, which is expressed in MMCs, meristemoid, and GMCs (Nadeau and Sack 2002). TMM is required for proper stomatal development. The cotyledons and leaves of the *tmm* mutant produce excessive stomata and stomatal clusters, whereas hypocotyls and stems are devoid of stomata (Geisler et al. 1998). TMM does not have an intracellular domain, suggesting that it is unlikely to transduce signals by itself. Genetic analysis of *tmm* and *erfs* indicated that the function of TMM depended on ERfs (Shpak et al. 2005). Protein immunoprecipitation showed that ERfs and TMM interacted *in vivo* (Lee et al. 2012). These data suggest that TMM might be a coreceptor for ERfs in stomatal regulation. However, how TMM participates in ERF signaling was not well understood.

Research revealed that the binding of EPF1 and EPF2 to ERfs needs TMM. For instance, overexpression of *EPF1* or *EPF2* in *tmm* mutants was unable to suppress stomatal formation (Hara et al. 2007; Hara et al. 2009). In isothermal titration calorimetry (ITC) assays, neither EPF1 nor EPF2 could bind to ERL1 in the absence of TMM, whereas when

TMM was present, these two ligands displayed greatly increased binding affinity to the ERL1-TMM complex (Lin et al. 2017). Protein crystal structures revealed that EPF1 and EPF2 interacted with ERfs via their N-terminal loops and β -sheets (Lin et al. 2017). When ERfs and TMM formed a complex, the N-terminus of TMM interacted with the two terminal loops of EPF1 and EPF2, which increased the binding strength of these two ligands to ERfs (Lin et al. 2017). Based on crystal structures of ERf-TMM-EPF1 protein complexes, amino acid residues S3, G13, and P50 on EPF1 were predicted to be important for the ligand-receptor interaction. In this study, *Arabidopsis* seedlings were treated with mutated EPF1 ligands. Results showed that when S3, G13, or P50 was mutated, the ability of EPF1 to regulate stomatal development was reduced. The structural analysis revealed that ERfs and TMM formed extensive contacts via their extracellular domains (Lin et al. 2017). ERL1^{E114}, TMM^{L281}, and TMM^{E379} were predicted to be involved in the interaction of ERL1 and TMM (Lin et al. 2017). In this study, it is shown that TMM^{E379R} failed to restore stomatal clustering phenotype of the *tmm* mutant in cotyledons, indicating an impaired function of TMM with this substitution. However, the other two mutations, ERL1^{E114} and TMM^{L281}, did not disturb the function of ERL1 and TMM in vivo, respectively.

Taken together, my work supports our protein crystal structures and reveal residues that are important for the function of EPF1 and TMM.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* ecotype Columbia was used as the wild type (wt). *tmm* (CS6140) was acquired from the *Arabidopsis* Biological Resource Center (Ohio State University). The *erl1 erl2* mutant used in the study have been described previously (Shpak et al. 2004). Seedlings were grown on Murashige and Skoog (MS) plates supplemented with 1% (w/v) sucrose. Plants were grown in a soil mixture with a 1:1 ratio of Promix PGX (Premier Horticulture, Inc.) and vermiculite (Pametto Vermiculite Co.) supplemented with

Miracle-Gro (Scotts) and ~3.5 mg/cm³ Osmocoat 15-9-12 (Scotts) at 20°C under long-day conditions (18 h light/6 h dark).

Plasmid construction and plant transformation

To construct a plasmid carrying *proTMM:TMM-3XHA* (pESH 730), the *TMM* coding region was cloned behind 540 bps of the *TMM* promoter sequence and in-frame with the 3×hemagglutinin (HA) tag (YPYDVPDYAGAYPYDVPDYAQLYPYDVPDYA) into the pZP222 vector. The 35S terminator was placed after the stop codon of 3xHA. The L281D and E379R substitutions were introduced into *TMM* by the overlap extension PCR using pESH730 as a template. The amplified fragment was digested with RsrII and PstI, inserted into pESH 730, and sequenced. The plasmids carrying *proTMM:TMM(L281D)-3XHA* and *proTMM:TMM(E379R)-3XHA* were named pESH 731 and pESH 732 respectively.

To construct a plasmid carrying *proERL1:ERL1-YFP* (pESH 702), the *ERL1* coding region was cloned behind the 4.1 kbs *ERL1* promoter sequence and in-frame with a YFP tag into the pZP222 vector. The 35S terminator was placed after the stop codon of YFP. The E114R and N117R substitutions were introduced into *ERL1* by the overlap extension PCR using pESH702 as a template. The amplified fragment was digested with KpnI and BamHI, inserted into pESH 702, and sequenced. The plasmids carrying *proERL1:ERL1(E114R)-YFP* and *proERL1:ERL1(N117R)-YFP* were named pESH 703 and pESH 704 respectively.

The described plasmids were introduced into an *Agrobacterium tumefaciens* strain (GV3101/ pMP90) by electroporation and then into *tmm* mutants or *er erl1 erl2* mutants by floral dip (Clough and Bent 1998). The transgenic plants were selected on MS plates containing gentamicin.

Peptide bioassay

One day after germination (DAG), wt *Arabidopsis* seedlings were transferred to MS liquid medium containing 1% (w/v) sucrose, 40 mg/mL timentin, and 10 µM EPF1 or mutated EPF1. For the competition assay of EPF1 and EPFL9, 10 µM EPF1 was used, the

concentration of EPFL9 was raised from 0 to 4 μ M. After 5 days of incubation at 20°C under long-day conditions (18 h light/6 h dark), seedlings were fixed, and stomata were measured by differential interference contrast (DIC) microscopy. EPF1 and EPFL9 Ligands were provided by Dr. Lin, Guangzhong and Dr. Chai, Jijie.

DIC microscopy

Seedlings were fixed overnight with ethanol: acetic acid (9:1 [v/v]). After fixation, samples were rehydrated with an ethanol series to 30% (v/v) ethanol and cleared in chloral hydrate solution (chloral hydrate: water: glycerol, 8:1:1 [w/v/v]). Microscopy was performed using Nikon Eclipse 80i microscope with DIC optics. Images were obtained with a 12-megapixel cooled color DXM-1200c (Nikon) camera. Stomata were measured on the abaxial side of cotyledons and on stems. Stomatal index was calculated using the formula: (number of stomata/total number of epidermal cells) $\times$ 100. Stomatal clustering was calculated using the formula: (number of clustered stomata/total number of stomata) $\times$ 100.

Quantitative reverse transcription PCR (RT-qPCR)

6 DAG *Arabidopsis* seedlings were ground to fine powder in liquid nitrogen. Total RNA was isolated using the Spectrum Plant RNA Isolation Kit (Sigma-Aldrich). The total RNA was treated with RNase-free RQ1 DNase (Promega). The first-strand complementary DNA was synthesized with oligo (dT) primers using a ProtoScript M-MuLV Taq RT PCRkit (New England Biolabs). Quantitative PCR was performed with a MyiQ Real-Time PCR Detection System (Bio-Rad) using Sso Fast EvaGreen Supermix (Bio-Rad). Each experiment contained three biological replicates and three technical replicates and was performed in a total volume of 10 μ L with 2.5 μ L of 50 times diluted cDNA. Cycling conditions were as follows: 3 min at 95°C; then 40 repeats of 15 s at 95°C, 30 s at 58°C for 3 $\times$ HA-tagged *TMM* and 59.3°C for *ACTIN2*, and 10 s at 72°C; followed by the melt-curve analysis. The cycle threshold values were calculated using the iQ5 software (Bio-Rad). The fold change of gene expression was calculated using relative quantification by the delta-delta Ct algorithm ($2^{-\Delta\Delta C_t}$).

$\Delta\Delta CT$). *ACTIN2* expression was used as the internal control. Primers used are listed as follow:

ACTIN2-F: 5'-CGGTGGTTCCATTCTTGC-3';

ACTIN2-R: 5'-GGACCTGCCTCATCATACTC-3'

3×HA TMM-F: 5'-TACGATGTTCTGACTACGCTG-3'

3×HA TMM-R: 5'-TTGCGGACTCCTCCTCTAGG-3'

Preparation of membrane proteins and protein immunoblot analysis (modified from Lee et al. 2012)

12 DAG *Arabidopsis* seedlings were ground to fine powder in liquid nitrogen and solubilized with 3× (w/v) extraction buffer (100 mM Tris-HCl at pH 8.8, 150 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM NaF, 1 mM PMSF, 1:1000 complete protease inhibitor cocktail [Sigma]). The extracts were sonicated on ice and centrifuged at 5000 rpm for 10 min at 4°C. The supernatants were ultracentrifuged at 32,000 rpm for 1 hr at 7°C to obtain the membrane fraction as precipitate. The pellet was resuspended in membrane solubilization buffer (100 mM Tris-HCl at pH 7.3, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM NaF, 1 mM PMSF, 1:1000 complete protease inhibitor cocktail) by gently pipetting on ice. The solution was ultracentrifuged again at 32,000 rpm for 1 hr at 7°C. The supernatants contained the membrane proteins. The isolated membrane proteins were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane (Amersham) for immunoblot analysis using polyclonal rabbit anti-HA (H6908, Sigma-Aldrich) antibody as the primary antibody at a dilution of 1:500, following by the secondary HRP conjugated goat anti-rabbit IgG antibody (Pierce) at a dilution of 1:5000. The detection of 3×HA-tagged TMM was performed with SuperSignal West Pico Rabbit IgG detection kit (Pierce).

Results

S3 is important for the function of EPF1

EPF1, EPF2, and EPFL4 form conserved interactions with ERfs via their N-terminal “GS” motifs (Lin et al. 2017). The serine residue in the “GS” motif seems to be crucial for the

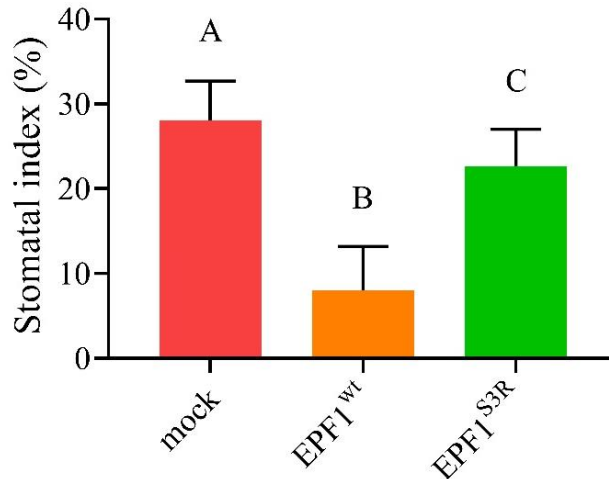


Figure 2.1 EPF1^{S3R} displayed reduced function. Seedlings treated with EPF1^{S3R} produced significantly more stomata on the abaxial side of cotyledons than wt EPF1 treated seedlings. Data is shown as mean + SD. N = 15-20. Significance was analyzed by one-way ANOVA. The p value of EPF1^{wt} vs EPF1^{S3R} was < 0.0001.

ligand-receptor interaction, because this residue forms three hydrogen bonds with neighboring residues on ERfs (Lin et al. 2017). We hypothesized that if this serine residue was mutated, the function of ligands would be reduced, and seedlings treated with the mutated ligands would have more stomata than seedlings treated with non-mutated ligands. This serine residue on EPF1 was replaced by arginine. Arginine has a long and positively charged side chain, which was predicted to disrupt the interactions formed between EPF1^{S3} and ERfs. As expected, wt EPF1 treated seedlings produced significantly fewer stomata than mock treated seedlings, while EPF1 containing the S3R substitution was unable to suppress stomatal formation as efficiently as wt EPF1 ligands (Figure 2.1). This indicates that the S3R substitution reduced the function of EPF1.

G13 and P50 are important for the function of EPF1

The binding of EPF1 to ERL1 requires TMM (Lin et al. 2017). The interaction of TMM and ERL1 creates an additional binding site for EPF1. The two terminal loops of EPF1 interact with the N-terminus of TMM (Lin et al. 2017). Glycine 13 on the N-terminal loop of EPF1 and proline 50 on the C-terminal loop were mutated to alanine and arginine,

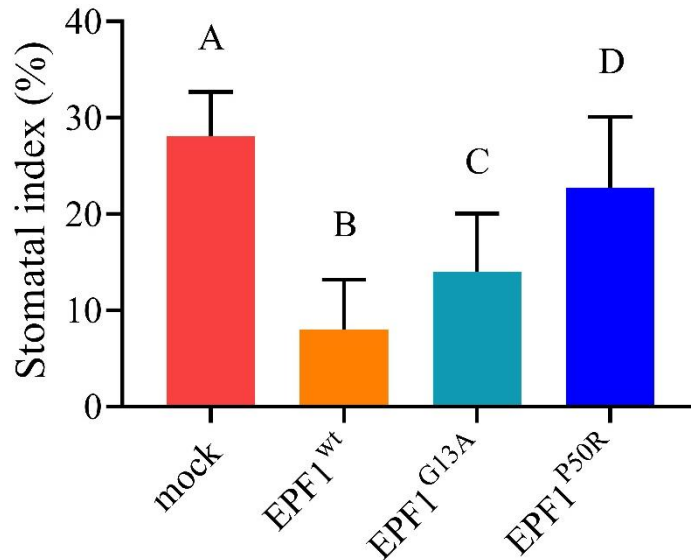


Figure 2.2 EPF1^{G13A} and EPF1^{P50R} displayed reduced function. Seedlings treated with EPF1^{G13A} or EPF1^{P50R} produced significantly more stomata on the abaxial side of cotyledons than wt EPF1 treated seedlings. Data is shown as mean + SD. N = 17-20. Significance was analyzed by one-way ANOVA. The p value of EPF1^{wt} vs EPF1^{G13A} was < 0.05. The p value of EPF1^{wt} vs EPF1^{P50R} was < 0.0001.

respectively. Based on crystal structures, we predicted that these two substitutions would compromise the interaction between TMM and EPF1. As expected, seedlings treated with EPF1 ligands containing either the G13A substitution or the P50R substitution had significantly more stomata than seedlings treated with wt EPF1 ligands, indicating that both substitutions reduced the function of EPF1 (Figure 2.2). However, in comparison to the P50R substitution, the G13A substitution exhibited a weaker effect on impairing the function of the ligand (Figure 2.2). The interactions between TMM and ERL1 are mediated by nonpolar contacts (Lin et al. 2017). Glycine has a smaller side chain than alanine, but both residues have nonpolar side chains. This may be the reason why the G13A substitution had a weak effect on the function of EPF1. However, because this subtle change did impact the activity of EPF1, G13 is indeed important for EPF1 to function.

E379 is important for the function of TMM

The extracellular domains of ERL1 and TMM formed right-handed superhelical structures (Lin et al. 2017). Almost the entire inner-face of TMM extracellular domain interacted with the convex side of ERL1 extracellular domain (Lin et al. 2017). ERL1^{E114}, TMM^{L281}, and TMM^{E379} were predicted to be involved in the interaction of TMM and ERL1 (Lin et al. 2017). Substitution of either one of these three residues disrupted the binding of ERL1 and TMM extracellular domains in vitro (Lin et al. 2017).

I generated transgenic *Arabidopsis* plants and investigated the effects of ERL1^{E114R}, TMM^{L281D}, and TMM^{E379R} on stomatal formation. Transformation of the *tmm* mutant with wt *TMM* restored the stomatal clustering phenotype in cotyledons, whereas plants transformed with *TMM*^{E379R} were indistinguishable from *tmm* mutants (Figure 2.3). The phenotype displayed by *TMM*^{E379R} transformed plants could be caused by poor expression of the transgene. To test this hypothesis, RT-qPCR was performed. *TMM* transcripts were detected in both *TMM* and *TMM*^{E379} transformed plants at a similar level (Figure 2.4A). To evaluate if *TMM* transcripts were successfully translated, membrane protein fraction was isolated and immunoblot analysis of 3×HA-tagged TMM was performed. TMM proteins existed in all transgenic lines but not in the *tmm* mutant (Figure 2.4B). These results indicate that glutamate 379 is important for the function of TMM in vivo. However, both *ERL1*^{E114R} transformed *erl1 erl2* mutants and *TMM*^{L281D} transformed *tmm* mutants had their stomatal clustering phenotype rescued in cotyledons (Figure 2.5). This indicates that substitutions of ERL1^{E114R} and TMM^{L281D} did not impair the function of ERL1 and TMM in vivo.

EPFL9 suppresses the function of EPF1 in vivo

EPFL9 competes with EPF2 for binding with ER, thus promoting stomatal formation (Lee et al. 2015). In vitro protein pull-down assay showed that EPFL9 also competed with EPF1 for binding to ERL1 (Lin et al. 2017). To see if the competitive binding of EPFL9 and EPF1 with ERfs happens in vivo, wt seedlings were cotreated with EPF1 and increasing concentrations of EPFL9. EPF1 alone inhibited stomatal formation, while simultaneous treatment of EPF1 with increasing concentration of EPFL9 resulted in increased stomatal

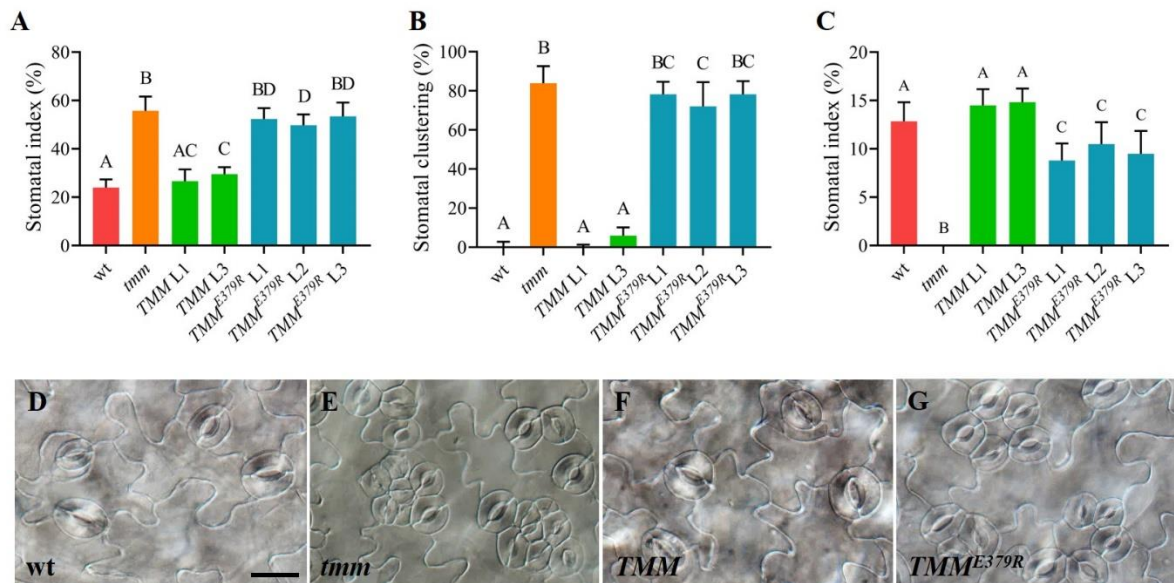


Figure 2.3 The E379R substitution reduced the function of TMM. (A, B) Stomatal index and stomatal clustering were analyzed on the abaxial side of 14-day-old cotyledons of wt, *tmm* mutants, and *tmm* mutants transformed with wt *TMM* (two independent lines were analyzed) or *TMM^{E379R}* (three independent lines were analyzed). (C) Stomatal index was analyzed on the mature stems of wt, *tmm* mutants, and *tmm* mutants transformed with wt *TMM* or *TMM^{E379R}*. Values are mean + SD. N = 18-30. (A-C) Significance was analyzed by one-way ANOVA. p value < 0.05. (D-G) DIC images of the abaxial side of 14-day-old cotyledons. Bar = 50 μ m.

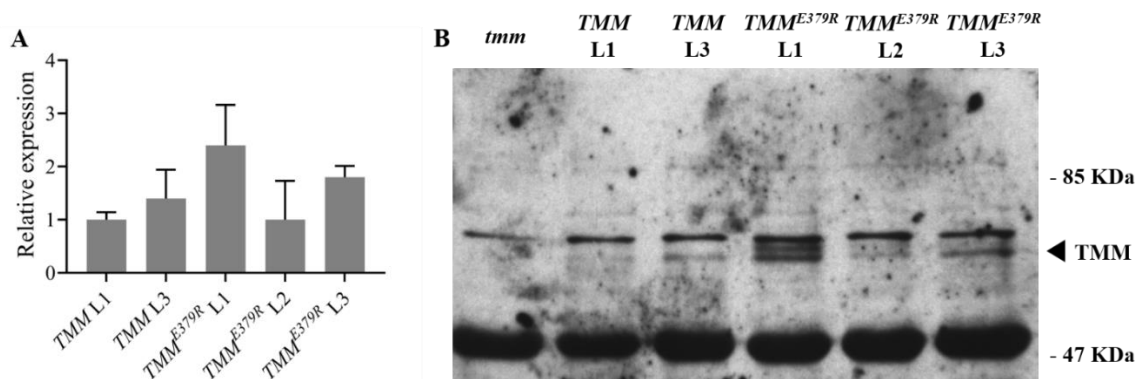


Figure 2.4 Analysis of *TMM* expression in transgenic plants. (A) RT-qPCR analysis of *TMM* expression in 6 DAG *tmm* seedlings transformed with wt *TMM* or *TMM^{E379R}*. Data shown are mean + SD. (B) Immunoblot analysis of membrane fraction of protein extracts from 12 DAG *tmm* mutant seedlings and *tmm* seedlings transformed with wt *TMM* or *TMM^{E379R}*. The black arrowhead indicates the band of *TMM* protein.

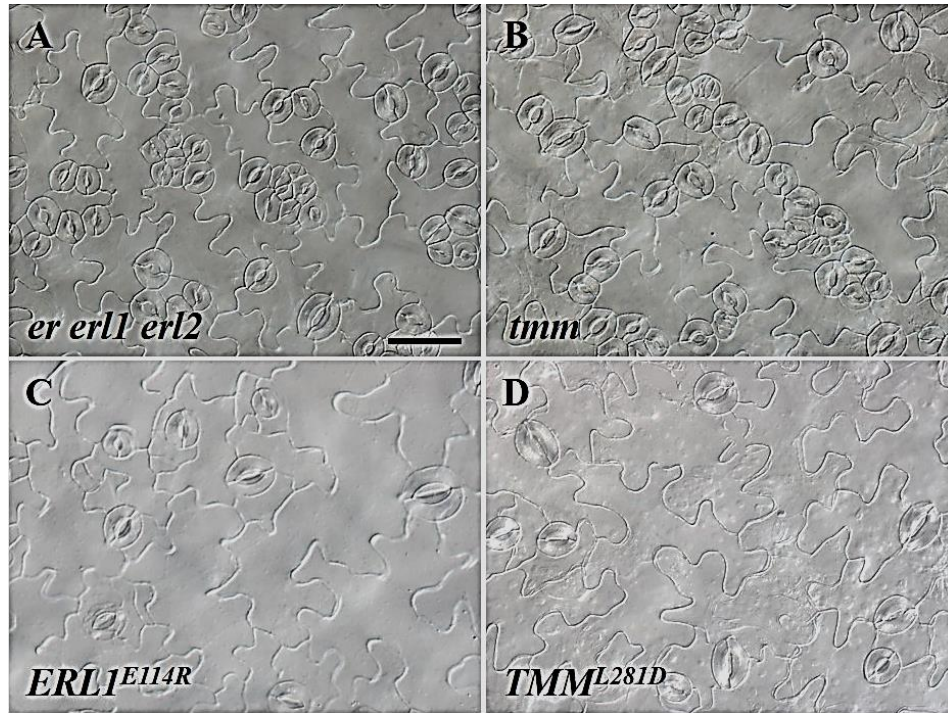


Figure 2.5 *ERL1^{E114R}* and *TMM^{L281D}* rescued the stomatal clustering phenotype of the *er erl1 erl2* and the *tmm* mutants, respectively. (A-D) DIC images of the abaxial side of 14-day-old cotyledons. (A) The *er erl1 erl2* mutant. (B) The *tmm* mutant. (C) The *er erl1 erl2* mutant transformed with *ERL1^{E114R}*. (D) The *tmm* mutant transformed with *TMM^{L281D}*. Bar = 50 μ m.

formation and stomatal clustering (Figure 2.6). My results indicate that EPFL9 competes with EPF1 for binding with ERfs in vivo, and EPFL9 suppresses the function of EPF1.

Discussion

Stomata control gas exchange between plants and the atmosphere. Hence, proper stomatal development is crucial for plant growth and productivity and is also important for the global water and carbon cycles. Studying how stomatal development is regulated can help us gain better understanding of how to manage plant growth and how to improve plant productivity.

In *Arabidopsis*, stomatal development is regulated by ERfs and their ligands EPF1, EPF2, and EPFL9. The binding of EPF1 and EPF2 to ERfs requires TMM. It was unknown how ERfs bound with their ligands and why TMM acted as the coreceptor for ligand binding. Our structural study of ERf-TMM-EPF/ EPFL protein complexes revealed the mechanism of interaction between ERfs, EPF/ EPFLs, and TMM (Lin et al. 2017). The goal of my work was to test our crystal structures in vivo. S3, G13, and P50 of EPF1 were predicted to form contacts with residues on ERL1. E379 of TMM was predicted to be involved in the interaction of TMM and ERL1. My work demonstrates that by mutating these residues, the function of EPF1 and TMM was reduced in vivo, indicating these residues are important for EPF1 and TMM to regulate stomatal development. Stomatal development is regulated by both negative signal and positive signal. EPF1 and EPF2 repress stomatal formation, while EPFL9 promotes stomatal formation. Whether an MMC will become a meristemoid is controlled by the competition between EPF2 and EPFL9 (Lee et al. 2015). My work shows that EPFL9 suppresses the function of EPF1 in vivo. Since EPF1 controls the transition of meristemoid to GMC, my work provides the evidence showing that this cell fate transition is also regulated by the competition of EPF/ EPFL ligands.

Protein crystal structures show that EPF1, EPF2, and EPFL4 form conserved interactions with ERfs via their N-terminal “GS” motifs (Lin et al. 2017). When the serine residue in the “GS” motif of EPF1 was mutated, the interaction of EPF1 and the ERL1-TMM

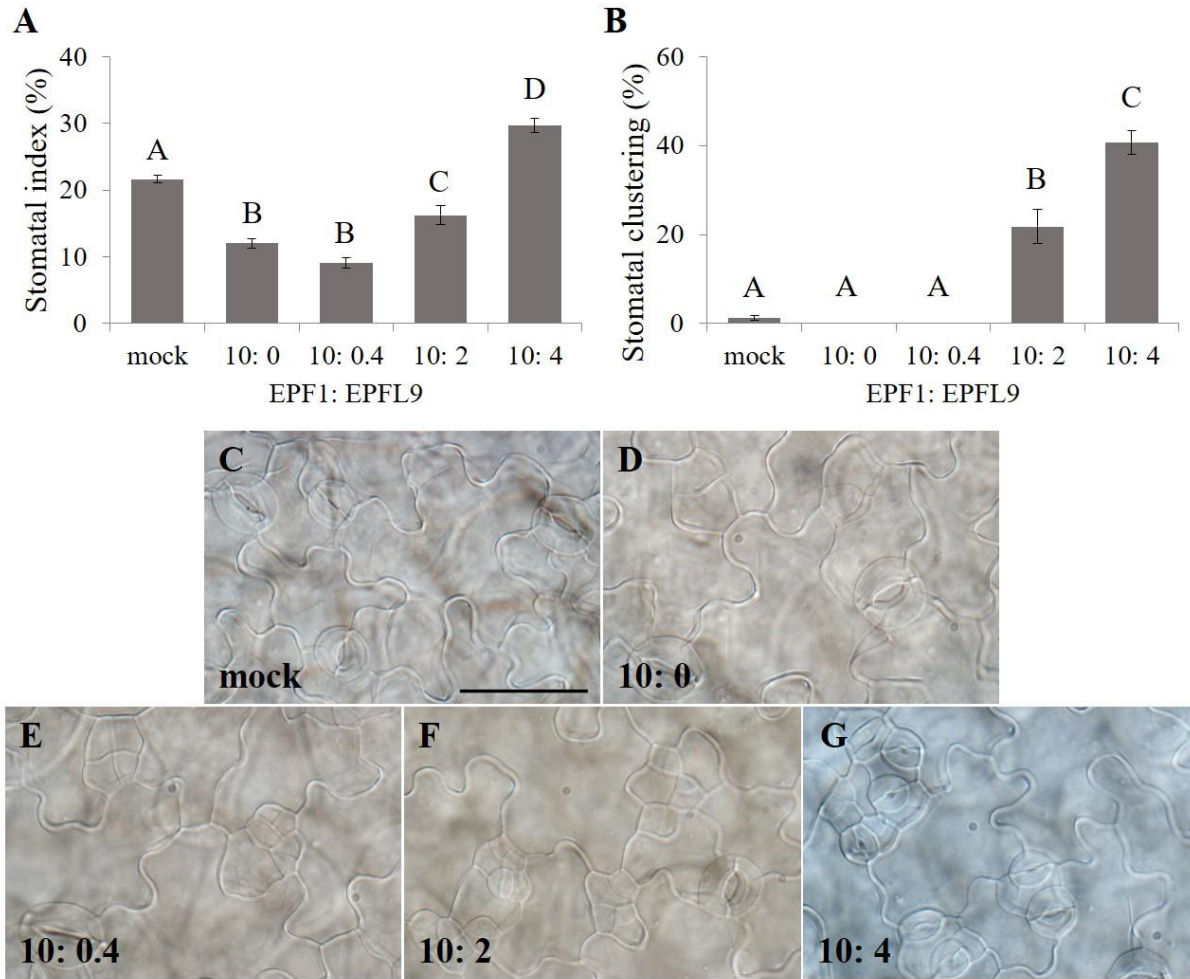


Figure 2.6 EPFL9 suppressed the function of EPF1 in vivo. (A-G) No ligand (mock). 10 μ M EPF1 (10: 0). 10 μ M EPF1 and 0.4 μ M EPFL9 (10: 0.4). (F) 10 μ M EPF1 and 2 μ M EPFL9 (10: 2). 10 μ M EPF1 and 4 μ M EPFL9 (10: 4). (A, B) Stomatal index and stomatal clustering were analyzed on the abaxial side of 6-day-old cotyledons of wt seedlings treated with or without ligands. Values are means $\pm$ SE. N=13-16. Significance was analyzed by one-way ANOVA. p value < 0.05. (C-G) DIC images of the abaxial side of 6-day-old cotyledons. Bar = 50 μ m.

protein complex was weakened (Lin et al. 2017). The mutated EPF1 had significantly reduced function. It was much less efficiently in repressing stomatal formation than wt EPF1. This “GS” motif is conserved in all 11 *Arabidopsis* EPF/ EPFL ligands and also exists in EPF/ EPFL ligands of other species (Rychel et al, 2010). We speculate that this “GS” motif mediates the specific interaction between EPFL ligands and ERfs. Because of this “GS” motif, ERfs can recognize EPFL ligands from other peptides.

EPF1 and EPF2 by their own cannot bind to ERL1. The binding of these two ligands to ERL1 requires the coreceptor TMM (Lin et al. 2017). In the presence of TMM, the binding affinity of EPF1 and EPF2 to the ERL1-TMM protein complex greatly increased (Lin et al. 2017). This is because the formation of the ERL1-TMM protein complex creates an additional binding site for EPF1 and EPF2. The binding of TMM to ERL1 makes the N-terminal loop of TMM adjacent to the N-terminal and the C-terminal loops of EPF1 (Lin et al. 2017). TMM and EPF1 forms multiple nonpolar interactions between their loop regions. G13 or P50 are the two residues on EPF1 involved in the interaction of EPF1 and TMM. When either one of these two residues was mutated, the interaction between EPF1 and the ERL1-TMM protein complex was weakened (Lin et al. 2017). The mutated EPF1 exhibited decreased activity in repressing stomatal formation. However, the binding of EPFL4 and EPFL6 to ERL1 does not require TMM. TMM reduced the binding affinity of EPFL4 and EPFL6 to the ERL1-TMM protein complex (Lin et al. 2017). Protein sequence alignment revealed that residues on EPF1 and EPF2 that interacted with TMM did not exist in EPFL4 or EPFL6 (Lin et al. 2017). Since EPF/ EPFL ligands show sequence diversity in their terminal loops, we hypothesize that the terminal loops may help ERfs to distinguish different EPF/ EPFL ligands.

The *tmm* mutant produces excessive stomata and stomatal clusters in leaves but no stomata in stems. In the presence of TMM, stomatal formation in leaves is determined by the balance between the negative regulation from EPF1/2 and the positive regulation from EPFL9. However, in the absence of TMM, EPF1/2 cannot bind to ERfs, thus cannot repress stomatal formation. In stems, besides EPF1/2, EPFL4/6 might also participate in the negative regulation on stomatal formation. EPFL4 and EPFL6 are expressed in the endodermis of stems (Abrash et al, 2011). These two ligands might move from the endodermis to the

epidermis, where they bind to ERfs and trigger signals to repress stomatal formation (Abrash et al, 2011). However, when TMM is present, EPFL4/6 cannot efficiently bind to ERfs, due to low binding affinity. Stomatal formation is still mainly regulated by EPF1/2 and EPFL9. In contrast, when TMM is absent, EPF1/2 no longer bind to ERfs, and the binding affinity of EPFL9 to ERfs decreases, whereas the binding affinity of EPFL4/6 to ERfs increases (Lin et al. 2017). These changes expose epidermal cells to overwhelmingly negative regulation, causing no stomata to form in *tmm* stems. Thus, the role of TMM is to help ERfs distinguish different EPF/ EPFL ligands.

ERL1^{E114} and TMM^{L281} are the two residues predicted to participate in the interaction of TMM and ERL1. When these two residues were mutated, ERL1 and TMM could no longer interact in vitro. However, ERL1 and TMM harboring mutation at these two sites were functional in vivo. A possible explanation for these results would be that these two single-point mutations were unable to disrupt ERL1-TMM interaction in vivo, due to the extensive contacts between these two proteins (Lin et al. 2017). *tmm* mutants transformed with *TMM*^{E379R} did not show restored stomatal formation in cotyledons. Intriguingly, these plants had stomatal defect partially recovered in stems (Figure 2.3C). These suggest that the mutation of TMM^{E379R} might impair both the binding of EPF1/2 and EPFL4/6 to ERfs. If the loss-of-function of TMM^{E379R} in vivo was the consequence of TMM^{E379R} was unable to interact with ERfs, as shown in protein pull-down assay, the binding of EPFL4/6 to ERfs should be enhanced. In other words, *tmm* mutants transformed with *TMM*^{E379R} should show no stomata in stems. An interesting question raising here is how TMM interact with ERfs in vivo? It is possible that TMM^{E379R} might still interacts with ERfs in vivo and this contact might make TMM close enough to disrupt EPFL4/6 binding with ERfs.

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**CHAPTER 3: ERECTA FAMILY SIGNALING CONSTRAINS *CLAVATA3* AND
WUSCHEL TO THE CENTER OF THE SHOOT APICAL MERISTEM**

Abstract

The shoot apical meristem (SAM) is a reservoir of stem cells that gives rise to all post-embryonic above-ground organs in plants. The size of the SAM remains stable over time due to a precise balance of stem cell replenishment versus cell incorporation into organ primordia. The WUSCHEL/CLAVATA negative feedback loop is central to SAM size regulation. Its correct functioning depends on tight spatial regulation of *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*) expression in the SAM's central zone. A signaling pathway, consisting of ERECTA family (ERf) receptors and EPIDERMAL PATTERNING FACTOR LIKE (EPFL) ligands, restricts SAM width and promotes leaf initiation. While ERf receptors are expressed throughout the SAM, EPFL ligands are expressed only in the periphery of the meristem. To test whether ERfs regulate width of the SAM through interaction with the CLV3/WUS loop we created higher order mutants. Genetic analysis demonstrated that ERfs and CLV3 synergistically regulate the size of SAM and *wus* is epistatic to *erfs*. Furthermore, activation of ERf signaling by exogenous treatment of seedlings with EPFL4 and EPFL6 resulted in a rapid decrease of *CLV3* and *WUS* expression. Here we demonstrate that ERf-EPFL signaling inhibits expression of *WUS* and *CLV3* in the periphery of the SAM, confining them to the center. These findings establish the molecular mechanism for stem cell positioning along the radial axis.

Introduction

The shoot apical meristem (SAM) generates new organs throughout the life of a plant. As stem cells in the central zone of the dome-shaped SAM slowly divide, some of their progeny are displaced laterally into the peripheral zone and basally into the rib zone. Cells in the peripheral and rib zones rapidly divide, differentiate, and are incorporated into forming leaves, flowers, and stems. Even though cells are constantly dividing, the SAM size remains stable throughout development due to a tight balance of proliferation and incorporation of cells into new organs.

The principal regulator of SAM size is a negative feedback loop consisting of *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*). *WUS* is a homeodomain transcription factor that maintains the pool of stem cells; in its absence stems cells arise but are almost immediately used up for organ formation (Laux et al. 1996). *WUS* is expressed in the organizing center beneath the central zone, and the protein moves up into the central zone through plasmodesmata (Mayer et al. 1998; Brand et al. 2000; Schoof et al. 2000; Yadav et al. 2011; Daum et al. 2014). *CLV3* encodes a secreted peptide expressed in the central zone and perceived by plasma membrane localized receptors: *CLV1* and *CLV1*-related *BARELY ANY MERISTEM1* (*BAM1*), *BAM2*, and *BAM3* (Fletcher et al. 1999; DeYoung et al. 2006; Shinohara and Matsubayashi 2015). In the central zone, *WUS* binds directly to the promoter of *CLV3* and activates its expression while *CLV3*-activated signaling inhibits *WUS* expression (Brand et al. 2000; Schoof et al. 2000; Yadav et al. 2011), forming a regulated feedback loop.

One of the central questions to understanding the meristem is how the spatial expressions of *CLV3* and *WUS* are established and maintained in the face of the continual turnover of cells. Previous studies have elucidated key mechanisms underlying the apical-basal distributions of *CLV3* and *WUS*. The depth of the *WUS* expression domain is defined by opposing activity of *CLV3* and cytokinin: *CLV3* inhibits *WUS* expression while cytokinin signaling promotes it. Both signals are produced in the apical region of the meristem and form a diffusion gradient along the apical basal axis. Cytokinin is perceived in deeper tissue layers than *CLV3* which establishes *WUS* expression at a certain distance from the surface of the SAM (Chickarmane et al. 2012). Confinement of *CLV3* expression to the region above the

WUS domain is dependent on HAM1, a transcription factor in the rib zone. Interaction with HAM1 prevents *WUS* from activating *CLV3* transcription, which restricts *CLV3* expression to the apical region of the SAM (Zhou et al. 2018). It remains, however, unclear why *WUS* and *CLV3* are expressed only around the central vertical axis of the SAM. Previous mathematical models have used implicit or explicit assumptions to define the lateral boundary that confines the expression of *WUS* and *CLV3* (Gruel et al. 2018; Zhou et al. 2018), but little is known about the actual existence of such a lateral signal. Here we present data showing that *ERECTA* family signaling restricts *WUS* and *CLV3* expression laterally, confining them to the center of the meristem and thereby providing a key mechanism for SAM maintenance.

ERECTA, *ERECTA-LIKE 1 (ERL1)*, and *ERL2*, collectively called *ERfs*, encode plasma membrane localized leucine-rich repeat receptor-like kinases (Shpak et al. 2004). The activity of ERF receptors is regulated by a group of cysteine-rich peptides belonging to the EPIDERMAL PATTERNING FACTOR/EPF-LIKE (EPF/EPFL) family (Hara et al. 2007; Hara et al. 2009; Lee et al. 2012; Lin et al. 2017). A mitogen-activated protein kinase cascade consisting of YODA, MKK4/5/7/9, and MPK3/6 functions downstream of the receptors (Bergmann et al. 2004; Wang et al. 2007; Lampard et al. 2009; Meng et al. 2012; Lampard et al. 2014). ERF signaling controls various developmental processes including stomata formation, above-ground organ elongation, SAM size, leaf initiation, and phyllotaxy (Chen et al. 2013; Shpak 2013; Uchida et al. 2013). In the SAM three ERFs function redundantly with single and double mutants having no or extremely weak meristematic phenotypes. Altered meristem development can be observed in the *er erl1 erl2* mutant which has a wider vegetative SAM and forms fewer leaves at almost random divergence angles (Chen et al. 2013; Uchida et al. 2013). Recently we demonstrated that activity of ERFs in the SAM is controlled by four ligands: EPFL1, EPFL2, EPFL4, and EPFL6 that are expressed at the periphery of the SAM (Kosentka et al. 2019). Based on altered expression of DR5rev:GFP and PIN1pro:PIN1-GFP markers in *er erl1 erl2* we proposed that the decrease in leaf initiation might be a result of altered auxin distribution (Chen et al. 2013). But the cause of the increased meristem size has remained unknown. Since regulation of SAM size depends on the *CLV3/WUS* feedback loop, we investigated whether ERFs genetically interact with these two genes and alter their expression. Our findings identify a new regulatory circuit that

enables communication between the peripheral and the central zones and specifies the location and size of the stem cell population in the SAM.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis* (*Arabidopsis thaliana*) ecotype Columbia was used as the wild type. The following mutants used in the study have been described previously: *er-105 erl1-2 erl2-1* (Shpak et al. 2004), *epfl1 epfl2 epfl4*, *epfl1 epfl2 epfl6* and *epfl1 epfl2 epfl4 epfl6* (Kosentka et al. 2019), *clv3-9* (Nimchuk et al. 2015), *wus* null allele (SAIL_150_G06) (Sonoda et al. 2007). They are all in the Columbia background.

To create *clv3 er erl2*, *clv3 er erl1 erl2*, and *wus er erl1 erl2* plants *clv3-9* and *wus/+*, were crossed with *er erl1/+ erl2*. To create *wus clv3* plants *clv3-9* was crossed with *wus/+*. To create *wus clv3 er erl1 erl2* plants *clv3 er erl1/+ erl2* were crossed with *wus/+ er erl1/+ erl2*. The higher order mutants were identified in the subsequent generations based on the phenotype of plants. The homozygous status of *wus* and *erl1* when necessary were confirmed by genotyping as described previously (Sonoda et al. 2007; Kosentka et al. 2017). To create *clv3 epfl1 epfl2 epfl4 epfl6* plants *clv3-9* mutant was crossed with *epfl1/+ epfl2 epfl4 epfl6*. The *epfl* mutations were genotyped as described previously (Kosentka et al. 2019).

Plants were grown as described elsewhere (Kosentka et al. 2017) under an 18-h light/ 6-h-dark cycle (long days) at 21°C. For the analysis of the SAM size and leaf initiation seedlings were grown on modified Murashige and Skoog medium plates supplemented with 1% (w/v) sucrose. For all experiments, seeds were stratified for 2 d at 4°C before germination.

To analyze expression of genes after EPFL4 and EPFL6 treatment, *epfl1 epfl2 epfl4* mutants were grown on modified Murashige and Skoog medium plates for 5 days (3 DPG). Then 60 seedlings (EPFL4 treatment) or 10 seedlings (EPFL6 treatment) per biological replicate were transferred to 1 ml of a liquid Murashige and Skoog medium containing 10 µM of EPFL4 or EPFL6. The purification of EPFL4 and EPFL6 peptides has been described

previously (Lin et al. 2017). EPFL peptides were dissolved in 10 mM Bis-Tris, 100 mM NaCl, pH=6.0. For mock treatment, a buffer solution of equal volume was added to the medium (92.6 μ l in the EPFL4 experiment and 8.7 μ l in the EPFL6 experiment). For each treatment there were 3 biological replicates. EPFL4 and EPFL6 Ligands were provided by Dr. Lin, Guangzhong and Dr. Chai, Jijie.

Microscopy

To measure leaf initiation and the size of SAM, 1, 3, and 5 DPG seedlings were fixed overnight with ethanol: acetic acid (9:1 [v/v]). After fixation, samples were rehydrated with an ethanol series to 50% (v/v) ethanol and cleared in chloral hydrate solution. Chloral hydrate: water: glycerol 8:1:1 [w/v/v] solution contained KOH at 10mM concentration to prevent degradation of tissues due to high acidity of chloral hydrate. In our experience the acidity of chloral hydrate (Sigma-Aldrich) varies from batch to batch and the necessity to add KOH should be tested experimentally. Microscopic observations of meristematic regions by DIC microscopy were performed as described previously (Chen et al. 2013).

For sectioning, tissue samples were fixed overnight in acetic acid: ethanol (1:9) at RT, dehydrated with a graded series of ethanol, and infiltrated with polymethacryl resin Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) followed by embedding and polymerization in Technovit 7100. Seven-micrometer sections were prepared using a Leica RM-6145 microtome (Wetzlar, Germany). The tissue sections were stained with 0.02% toluidine blue O and observed under bright-field illumination. Pictures of older seedlings and the analysis of flower structure was done using a Leica MZ16 FA stereomicroscope.

qPCR analysis

Total RNA was isolated from the aboveground tissues of seedlings using the Spectrum Plant RNA Isolation Kit (Sigma-Aldrich). The RNA was treated with RNase-free RQ1 DNase (Promega). First-strand complementary cDNA was synthesized with LunaScript™ RT SuperMix Kits (New England Biolabs). Quantitative PCR was performed with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using Sso Fast EvaGreen Supermix (Bio-

Rad). Each experiment contained three technical replicates of three biological replicates and was performed in a total volume of 10 μ L with 4 μ L of 10x or 50x diluted cDNA. Cycling conditions were as follows: 3 min at 95°C; then 40 repeats of 10 s at 95°C, 10 s at 52°C for *ACTIN2* and *STM*; 10 s at 55°C for *WUS*; 10s at 57°C for *MP*; 10 s at 50°C for *CLV1*, *BAM1*, *BAM2*, and *BAM3*, and 10 s at 68°C, followed by the melt-curve analysis. Cycling conditions for *CLV3* were 3 min at 95°C; then 40 cycles of 95°C for 10 s, and 60°C for 10 s, followed by the melt-curve analysis. Primers for *ACTIN2*, *STM*, *WUS*, and *MP* (Chen et al. 2013) as well as for *CLV3* (Chiu et al. 2007) have been described previously. Primers for *CLV1*, *BAM1*, *BAM2*, and *BAM3* were as in (Nimchuk et al. 2015). The fold difference in gene expression was calculated using relative quantification by $2^{-\Delta\Delta CT}$ algorithm.

Results

ERf and CLV3 signaling synergistically restrict SAM size

Comparison of *clv3* and *er erl1 erl2* mutants suggests that while both ERfs and CLV3 control SAM size, they play dominant roles during different developmental stages. At one day post germination (DPG) the SAM of *er erl1 erl2* is considerably wider (105.1 ± 3.3 ; mean $\pm$ SE) than in the wild type (56.1 ± 1.7 ; $p < 1.2 \cdot 10^{-13}$ in student t-test) or in *clv3* (83.0 ± 1.4 ; $p < 4.2 \cdot 10^{-7}$) (Figure 3.1A, C), suggesting a key role for ERfs in restricting SAM size during embryogenesis. For the first five days after germination the wild type and *er erl1 erl2* SAMs do not substantially increase in width, while *clv3* SAM size continues to increase, indicating that post embryogenesis CLV3 signaling plays the primary role in SAM size maintenance (Figure 3.1A). The pathways also contribute differently to leaf initiation, with ERfs promoting leaf initiation and CLV3 slightly inhibiting it (Figure 3.1B).

The most dramatic phenotype is observed when both signaling pathways are deactivated. The *clv3 er erl1 erl2* mutants have a dramatically larger SAM immediately after germination (Figure 3.1A). Later, *clv3 er erl1 erl2* develops a massive SAM that is mostly incapable of organogenesis (Figure 3.1D, E). In rare occasions the mutant will form one or two leaves or produce structures resembling stigmas, but it never forms a stem even after

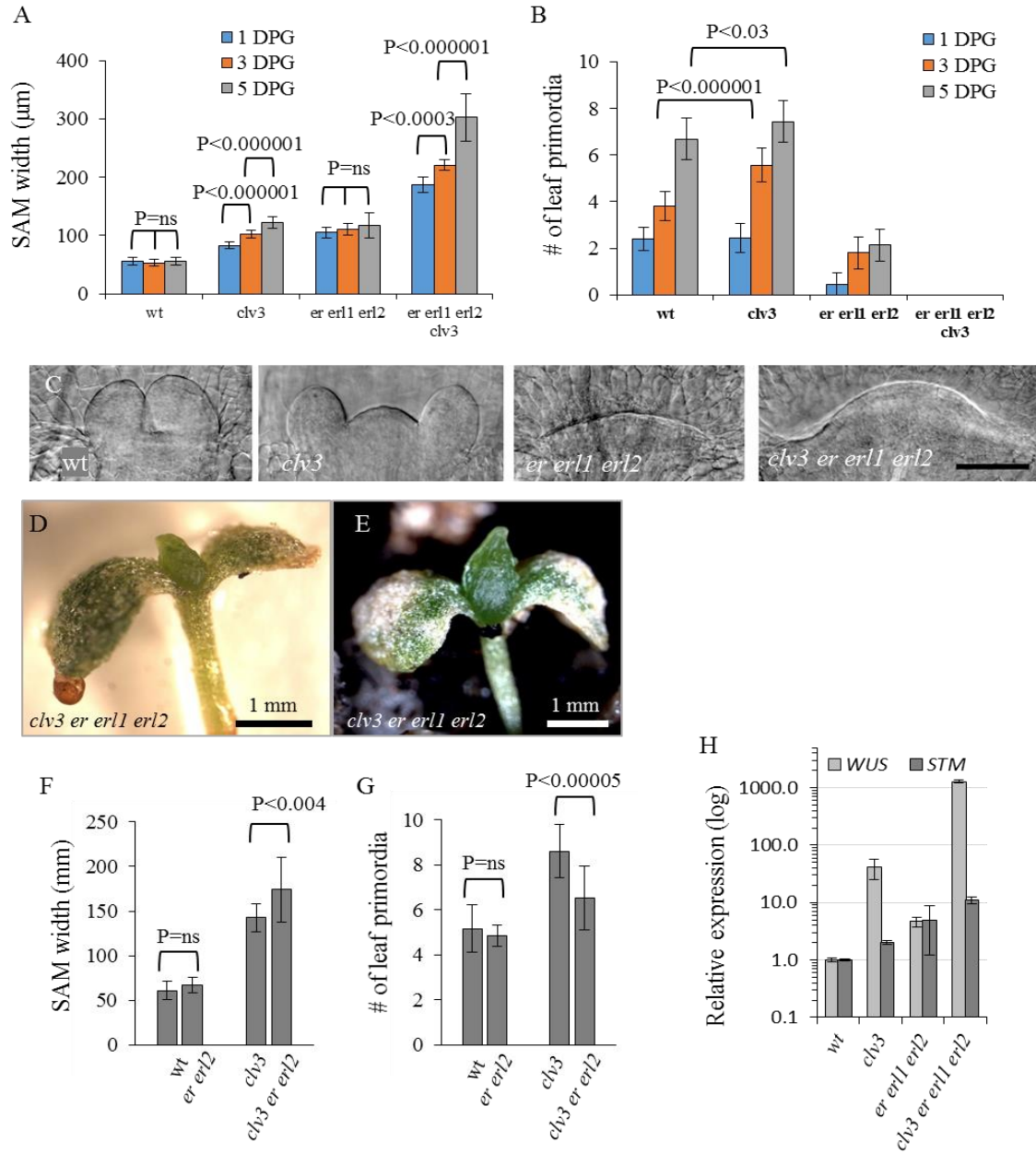


Figure 3.1 *CLV3* and *ERfs* synergistically regulate SAM size and leaf initiation. (A, B, F and G) Comparison of the SAM width and leaf initiation. The rate of leaf primordia initiation was determined by DIC microscopy of fixed samples. (A) primordium was defined as a bulge over 15 μm. Values are mean ± SE, N = 6-18. DPG, days post germination. An absence of bars in *er erl1 erl2 clv3* represent a complete absence of leaf primordia in that genotype at that age. The brackets represent results of statistical analysis using Student *t*-test. (C) The SAM of dark grown seedlings 1 DPG. All images under the same magnification. Bar = 50 μm. (D) 29 DAG *clv3 er erl1 erl2* plant. (E) 42 DAG *clv3 er erl1 erl2* plants. (H) qPCR analysis of *WUS* and *STM* in above-ground organs of 5 DPG seedlings of wild-type (wt) and mutants as indicated. The average of three biological replicates is presented. Error bars represent SD.

more than 40 days of growth (Figure 3.2A, B). The meristematic nature of the dome-like structure in *clv3 er erl1 erl2* is consistent with the presence of cells with dense cytoplasm and without chlorophyll in the outer cell layers (Figure 3.5A; Figure 3.2C). Moreover, the epidermal layer is composed of very small cells and the guard cells are absent, indicating the absence of differentiation (Figure 3.2D). Our data are consistent with the larger *clv3 er erl1 erl2* SAM in 10-day-old seedlings described previously (Kimura et al. 2018). The synergistic function of CLV3 and ERfs in the SAM is also evident in the *clv3 er erl2* mutant: while the *er erl2* mutant has a meristem indistinguishable from the wild type, these two mutations enhance the width of the *clv3* SAM, and *er erl2* reduces leaf initiation in the *clv3* background (Figure 3.1F, G). Finally, *CLV3* regulates SAM size and leaf initiation in concert with the meristematic ERf ligands *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6*. The size of the SAM is dramatically increased in *clv3 epfl1 epfl2 epfl4 epfl6* plants (Figure 3.3). Taken together, these findings indicate that during embryogenesis and after germination *ERf* and *CLV3* signaling pathways synergistically restrict SAM size. The extent of their individual contributions varies at different developmental stages. Before germination both contribute to SAM size maintenance with ERfs playing the primary role. After germination their roles switch with *CLV3* playing the dominant role and ERf signaling being auxiliary.

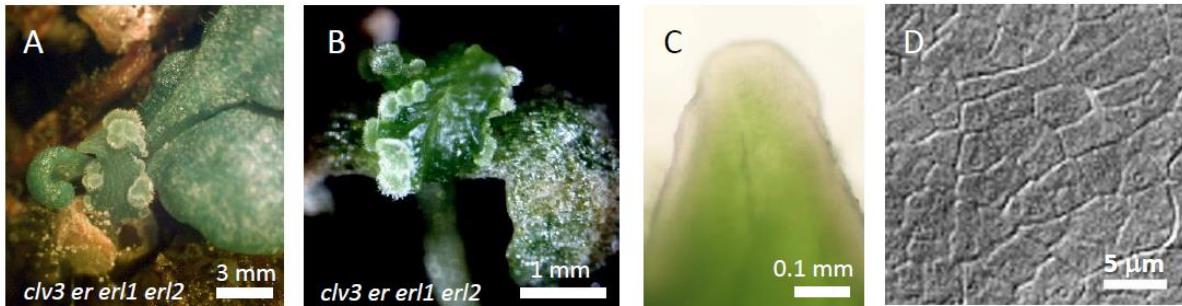


Figure 3.2 *CLV3* and *ERfs* synergistically regulate SAM size and leaf initiation. (A and B) Formation of papillae structures on the surface of the SAM in 32 DPG and 42 DPG *clv3 er erl1 erl2* plants, respectively. (C) A bright-field image of the *clv3 er erl1 erl2* SAM demonstrates an absence of the chlorophyll in the outer cell layers. (D) A DIC image of the epidermis of the dome like structure in the *clv3 er erl1 erl2* mutant.

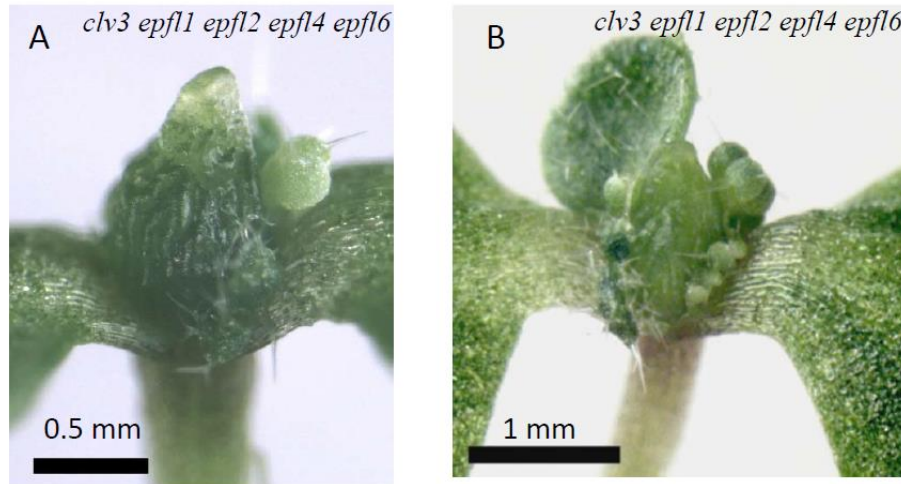
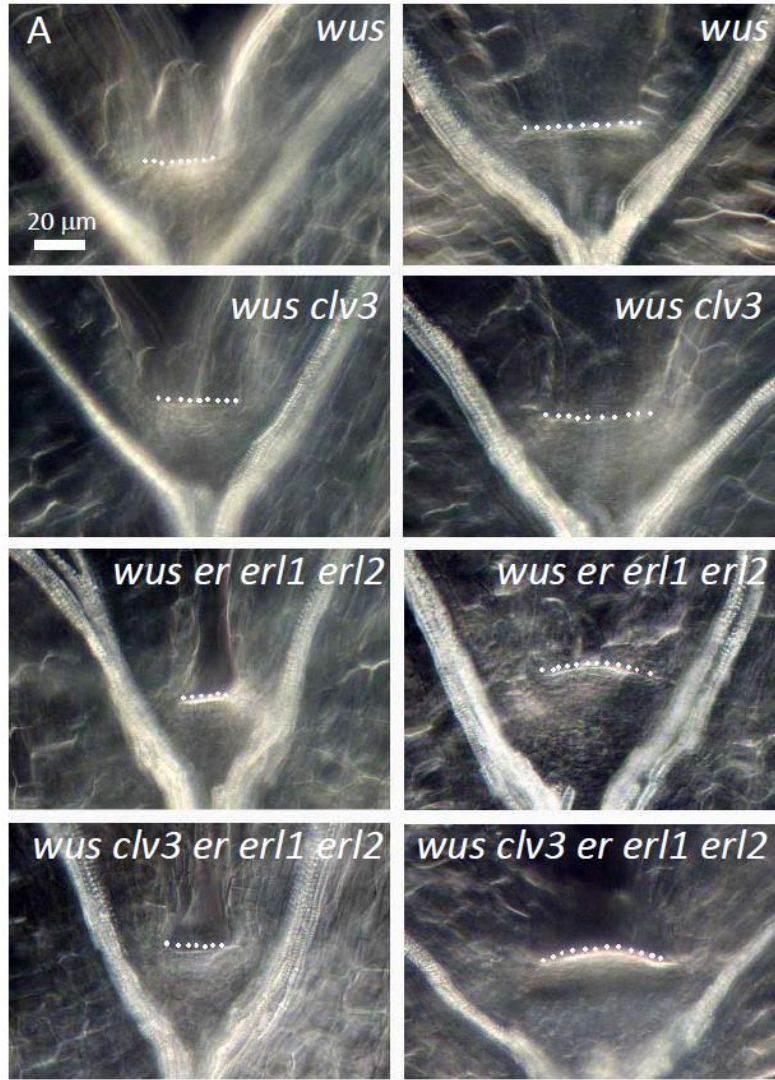


Figure 3.3 CLV3, EPFL1, EPFL2, EPFL4, and EPFL6 synergistically regulate SAM size. 19 DPG *clv3 epfl1 epfl2 epfl4 epfl6* seedlings. The phenotype of *clv3 epfl1 epfl2 epfl4 epfl6* is less severe compared to *clv3 erl1 erl2* (Figure 3.1D, E) with leaf and flower-like organs forming at the edge of the SAM and the petioles of cotyledons. Images taken by Daniel DeGennaro.

The *wus* mutation is epistatic to *erl1 erl2*

CLV3 is known to regulate meristem size by inhibiting expression of *WUS* (Brand et al. 2000; Schoof et al. 2000; Muller et al. 2006). While expression of *WUS* is increased in an *erl1 erl2* background, the increase is relatively moderate: 4-6x five DPG (Chen et al. 2013; Uchida et al. 2013) (Figure 3.1H). The significance of ERfs for regulation of *WUS* expression becomes more evident in the absence of CLV3 signaling. In 5 DPG *clv3 erl1 erl2* seedlings we observed up to a ~30x increase in *WUS* expression compared to the single *clv3* mutation and a ~1000x increase over the wild type (Figure 3.1H). The increase of *WUS* expression in *clv3 erl1 erl2* is unlikely to be a direct outcome of a bigger meristem since the other meristematic marker *STM* increases much more moderately: ~5.5x compared to *clv3* and 11x compared to the wild type. The large increase of *WUS* expression in *clv3 erl1 erl2* suggests that expression of *WUS* is synergistically regulated by CLV3 and ERfs.

To study genetic interactions between *ERfs* and *WUS* and to compare them with the genetic interactions of *CLV3* and *WUS*, we measured SAM size in *wus*, *wus erl1 erl2*, *wus clv3*, and *wus clv3 erl1 erl2* mutants at three and five DPG. While SAM width varied



B

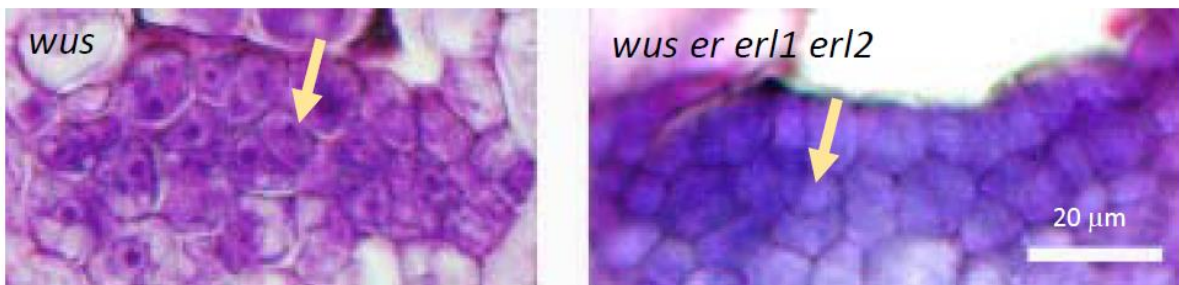


Figure 3.4 Meristematic phenotypes of 5 DPG *wus*, *wus clv3*, *wus er erl1 erl2*, and *wus clv3 er erl1 erl2* seedlings. (A) Variations of the SAM size in seedlings containing *wus* mutation. Both relatively small (on the left) and relatively big (on the right) meristems were observed in all four genotypes. All images under the same magnification. (B) Periclinal division in the L2 layer of *wus* and *wus er erl1 erl2* SAM as indicated by an arrow.

significantly in individual seedlings (Figure 3.4A), the four mutants were statistically indistinguishable (Figure 3.5B) suggesting that during early seedling growth *wus* is epistatic to both *clv3* and *er erl1 erl2*. This conclusion is supported by histological analysis: the shoot apices of *wus*, *wus er erl1 erl2*, and *wus clv3 er erl1 erl2* mutants did not have the classic dome-like SAM structure consisting of multiple layers of small, evenly shaped, and tightly packed stem cells (Figure 3.5A). In the mutants, the shoot apices were composed of only two layers of small cells with some of those cells dividing periclinally, a sign of premature differentiation (Figure 3.4B). A previous analysis of *wus er erl1 erl2* using 10 DPG seedlings indicated that its SAM is bigger than that of *wus* suggesting additive effects of ERf and WUS (Kimura et al. 2018). This conclusion was supported by the ability of *er erl1 erl2* mutations to partially rescue initiation of stamens and carpels in the *wus* background (Kimura et al. 2018). However, our more comprehensive data and analysis of *wus er erl1 erl2* does not support the hypothesis of additive ERf and WUS interactions. At ten DPG in many *wus er erl1 erl2* seedlings we observed a narrow region between forming leaf primordia (Figure 3.6). While in some seedlings the area between forming leaves was indeed enlarged, it did not contain stem cells with the characteristic dense cytoplasm (Figure 3.6). Based on morphology, cells in that region are differentiated: they are highly vacuolated, and some L2 layer cells divide in orientations other than anticlinal. We did occasionally observe meristem-like aggregations of small cells with dense cytoplasm; however, those structures were always small in diameter and asymmetrically localized, often at the axil of a leaf. These structures are either axillary meristems or leaf primordia arising of new meristematic regions or leaf primordia might be altered in *wus er erl1 erl2* compared to *wus*, there is no rescue of the central zone maintenance. Our analysis of *wus er erl1 erl2* flower structure in two-month old plants indicated that ERf family mutations were unable to rescue carpel or stamen initiation in the *wus* background (Table 3.1). While analyzing flower development we observed formation of stigma-like structures at the tips of sepals and the formation of stigma-like tissue in the area of the SAM in older plants, but in flowers that emerge soon after bolting we never observed the formation of carpels. Considering that we used the same alleles of *WUS* and *ERfs* we are not sure why we cannot reproduce the *wus er erl1 erl2* flower structure data described by Kimura

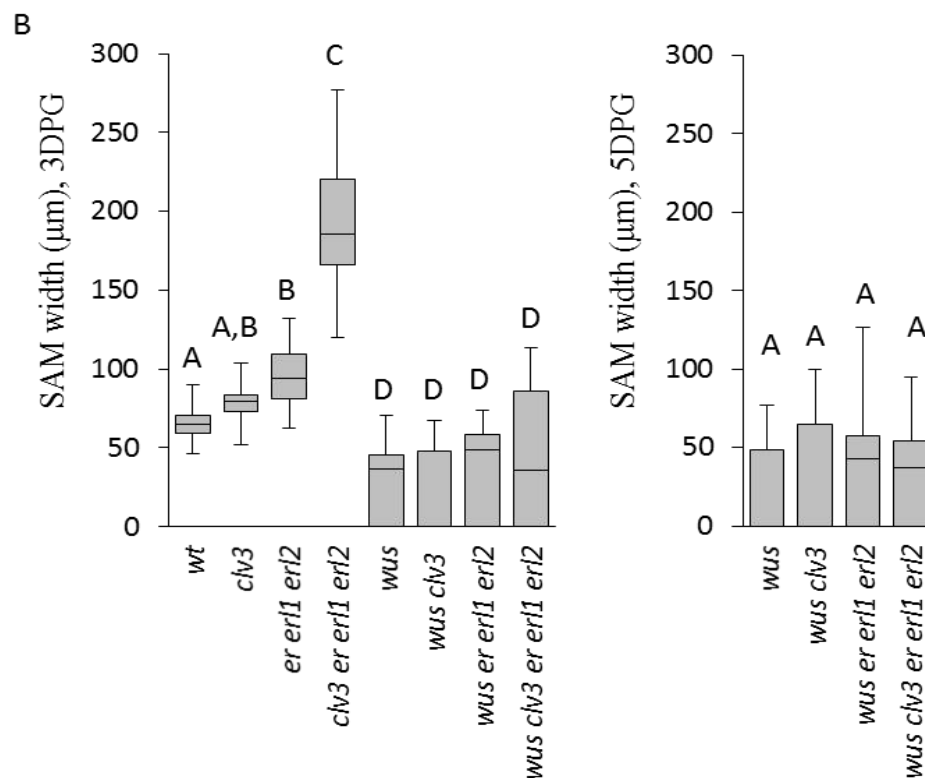
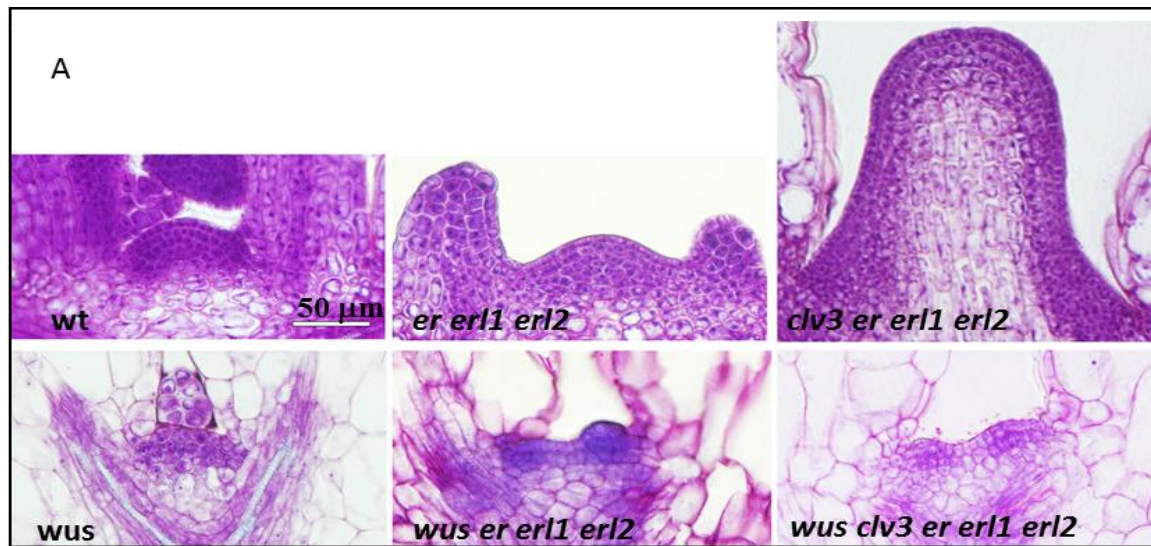


Figure 3.5 *wus* is epistatic to *er erl1 erl2*. (A) Median sections of shoot apices of 5 DPG seedlings. All images are under the same magnification. (B) SAM width measurements performed by DIC microscopy using 3 DPG (left) and 5 DPG (right) seedlings. N = 15-36. Error bars represent SD. Statistical analysis was done by one-way anova; ns, not significance.

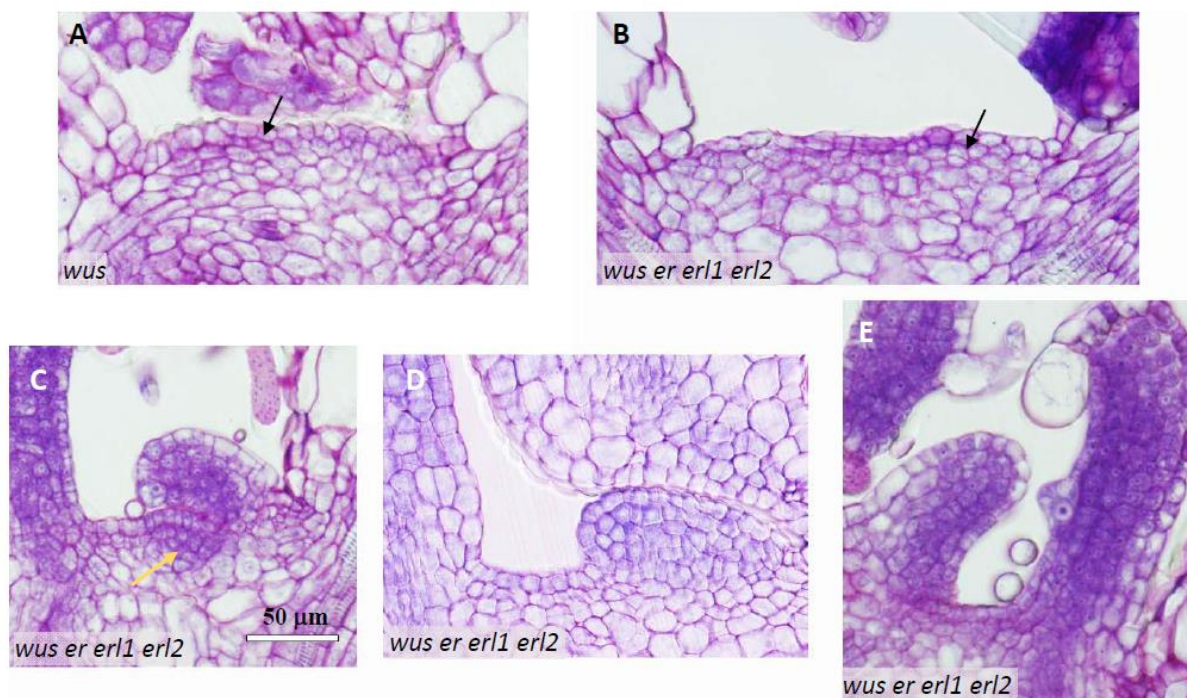


Figure 3.6 Meristematic phenotypes of 10 DPG *wus* and *wus erl1 erl2* seedlings. The area at the top of the hypocotyl is often similar in size and structure in *wus* (A) and *wus erl1 erl2* (B) seedlings with darkly stained stem cells being absent and periclinal cell divisions in L2 layer (indicated by a black arrows). Whenever leaf primordium initiation is observed in *wus erl1 erl2*, the adjacent “meristematic” region is narrow (C, D and E) with only rare occasions of stems cells (indicated by a yellow arrow) present (C).

Table 3.1 Number of flower organs. As indicated by average $\pm$ S.D. n indicates the number of flowers analyzed.

	Sepal	Petal	Stamen	Carpel	n
wt	4.0 $\pm$ 0.0	4.0 $\pm$ 0.0	6.0 $\pm$ 0.0	2.0 $\pm$ 0.0	10
<i>erl1 erl2</i>	4.0 $\pm$ 0.0	4.0 $\pm$ 0.0	6.0 $\pm$ 0.0	2.0 $\pm$ 0.0	16
<i>erl1 erl2</i>	3.8 $\pm$ 0.6	2.1 $\pm$ 1.0	5.1 $\pm$ 0.6	2.0 $\pm$ 0.0	22
<i>wus</i>	3.9 $\pm$ 0.8	2.9 $\pm$ 1.1	0.8 $\pm$ 0.9	0.0 $\pm$ 0.0	49
<i>wus erl1 erl2</i>	3.5 $\pm$ 0.8	3.1 $\pm$ 0.9	1.2 $\pm$ 1.1	0.0 $\pm$ 0.0	80
<i>wus erl1 erl2</i>	3.3 $\pm$ 1.2	1.6 $\pm$ 1.0	1.2 $\pm$ 1.1	0.0 $\pm$ 0.0	13

and colleagues (Kimura et al. 2018). In sum, our data indicate that *wus* is epistatic to *er erl1 erl2* in regulation of the SAM central zone width and in the flower meristem.

ERf signaling inhibits expression of *CLV3* and *WUS*

The expression of both *CLV3* and *WUS* are increased in the *er erl1 erl2* background, with *in situ* hybridization and promoter GUS fusions showing expansion of their expression in the lateral orientation (Chen et al. 2013; Uchida et al. 2013; Kimura et al. 2018). While *epfl1 epfl2 epfl4* and *epfl1 epfl2 epfl6* mutants exhibit only a very slight increase in SAM size (Kosentka et al. 2019), they also express *CLV3* at a higher level (Figure 3.7A). Is the increase of *WUS* and *CLV3* expression in *er erl1 erl2* a consequence of an enlarged SAM or does ERF signaling inhibit expression of these two genes? To answer this question, we treated *epfl1 epfl2 epfl4* seedlings exogenously with either the EPFL4 peptide or the EPFL6 peptide for 6 hours. qPCR analysis revealed a significantly decreased expression of *WUS* and *CLV3* in response to both peptides (Figure 3.7C). Several other genes that have altered expression in *er erl1 erl2* such as *STM* (Figure 3.1H), *MONOPTEROS (MP)* (Chen et al. 2013), and *CLV1* (Figure 3.8), did not change expression after the peptide treatment (Figure 3.7C), suggesting specificity in the downregulation of *CLV3* and *WUS*. The decrease in *WUS* and *CLV3* transcription levels was dependent on the presence of functional ERF receptors, since it was not observed when *er erl1 erl2* seedlings were treated with EPFL4 (Figure 3.7B). These data imply that *WUS* and *CLV3* are downstream targets of the ERF signaling pathway.

Interestingly, the *er erl1 erl2* mutant has reduced sensitivity to *CLV3* peptide (Kimura et al. 2018) suggesting that ERfs might have additional roles in regulation of the *CLV3* signaling pathway. The reduced sensitivity of the mutant to *CLV3* might be related to reduced expression of several *CLV3* receptors: *CLV1*, *BAM1*, and *BAM2* (Figure 3.8).

Discussion

Previously it has been noticed that *ERECTA* and *CLV3* function along different spatial axes: *CLV3* preferentially regulates meristem height and *ERECTA* regulates meristem

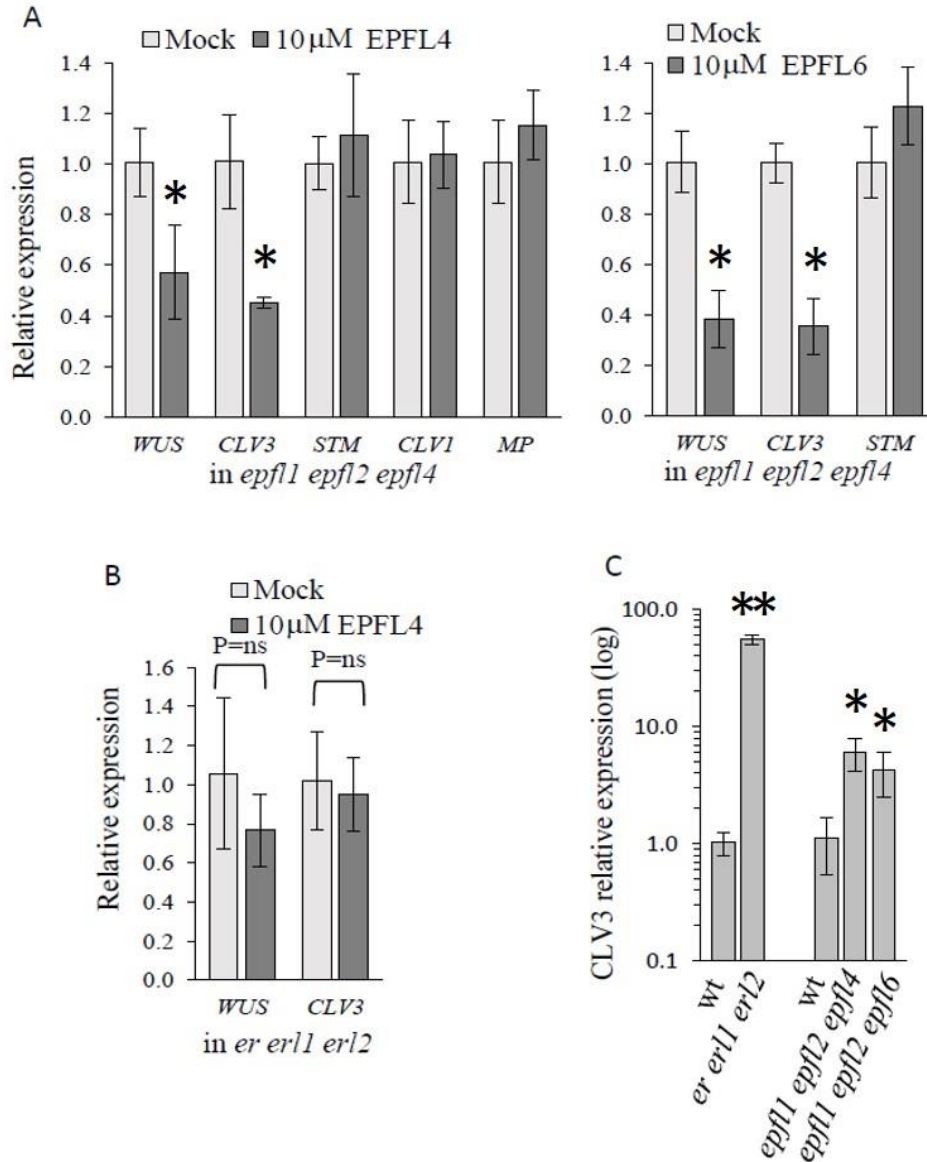


Figure 3.7 *WUS* and *CLV3* are targets of ERF signaling pathway. (A and B) Relative expression levels of selected mRNAs in above-ground organs of 3 DPG *epfl1 epfl2 epfl4* (A) or *er erl1 erl2* (B) seedlings 6 h after treatment with 10 μM EPFL4 or EPFL6 peptides compared to mock treatment. Expression significantly differs from mock ($P < 0.05$) is indicated by asterisks. ns, not significant. (C) *CLV3* relative expression levels in above-ground parts of 5DPG seedlings in genotypes as indicated. Data are shown as means $\pm$ SD. Expression significantly differs from the wt ($P < 0.05$) is indicated by single-asterisk, ($P < 0.005$) is indicated by double-asterisk. (A-C) *ACTIN2* was used as an internal control.

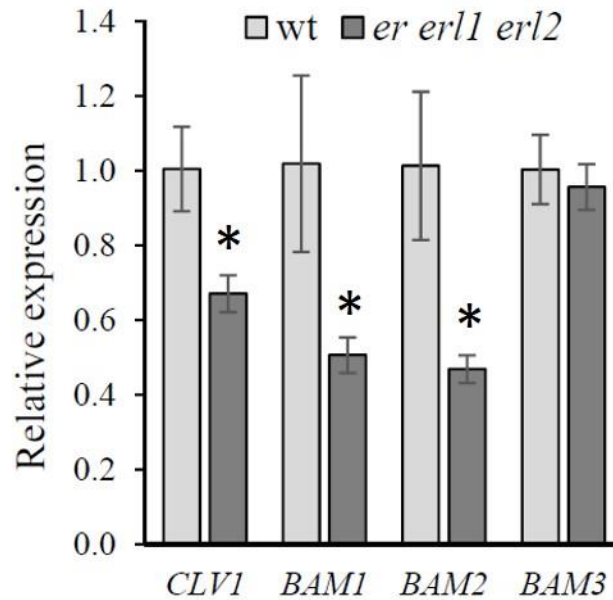


Figure 3.8 Expression of *CLV1*, *BAM1*, and *BAM2* is decreased in *er erl1 erl2*. Relative expression levels were analyzed in above-ground parts of 5 DPG seedlings in genotypes as indicated. Data are shown as means $\pm$ SD. Expression significantly differs from wt ($P < 0.05$) is indicated by asterisks. *ACTIN2* was used as an internal control.

width (Mandel et al. 2016). Four ligands that regulate activity of ERfs in the SAM are mostly expressed at the periphery of the meristem and are excluded from both the central zone and the organizing center (Kosentka et al. 2019). EPFL1 expression in the peripheral zone under the *KANADI* promoter fully rescues meristematic defects of the *epfl1 epfl2 epfl4 epfl6* mutant (Kosentka et al. 2019). In contrast, while *ERfs* are expressed throughout the SAM, their function in the center of the meristem is critical for SAM maintenance. We previously found that *ERECTA* expressed under the *CLV3* promoter rescues the meristematic defects significantly better compared to its expression under the *KANADI* promoter (Kosentka et al. 2019). These findings imply that ERfs restrict the width of the SAM by sensing EPFLs originating from the periphery of the SAM. The data suggest that ERfs restrict the width of the central zone by inhibiting expression of *CLV3* and *WUS* (Figure 3.9). This control is especially important during establishment of the SAM in embryogenesis. After germination *CLV3* signaling can partially substitute for ERfs in the lateral inhibition of *WUS* expression. However, if both signaling pathways are disrupted, as in the *er erl1 erl2 clv3* mutant, *WUS*

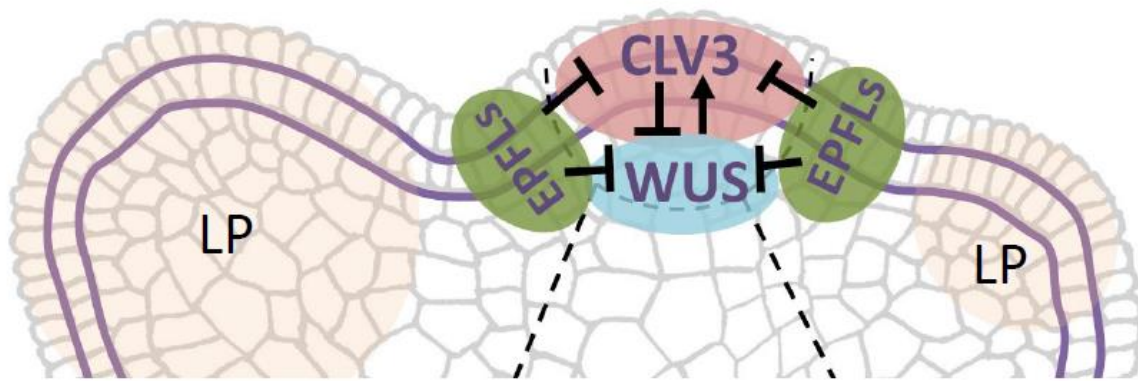


Figure 3.9 Model for the role of ERF signaling in regulation of shoot meristem maintenance. The EPFL signals originating from the periphery of the SAM activate ERF signaling cascade that inhibits expression of *CLV3* and *WUS* thus restricting the width of the central zone. LP: leaf primordium.

expression becomes rampant and the SAM expands without restraint. Increased expression of *CLV3* in the L1 layer of *erl1erl2* and *wus erl1 erl2* (Kimura et al. 2018) and the importance of ERfs in suppressing *CLV3* expression in the periphery of the meristem suggest that an as-yet unidentified signal induces *CLV3* expression in the epidermis, consistent with a previously proposed model (Gruel et al. 2016). ERF and WUS modulate that expression by creating a gradient of *CLV3* expression with the strongest expression in the center of the SAM and falling off in the periphery. The decrease of *CLV3* expression in the periphery of the meristem, which we propose is due to ERF signaling, is important since uniform expression of that gene throughout L1 leads to a complete shutdown of WUS expression (Lenhard and Laux 2003).

These findings significantly advance our understanding of the mechanisms controlling SAM size and morphology. They identify ERF signaling as an important modulator of *CLV3* and *WUS* expression domains, and consequently of the stem cell reservoir volume.

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CHAPTER 4 CONCLUSIONS

Chapter 2

Our structural study demonstrates the mechanism of interactions between ERfs, TMM, and EPF/ EPFL ligands (Lin et al. 2017). EPF1, EPF2, EPFL4, and EPFL6 bind to ERfs via their β -sheets and the “GS” motifs in their N-terminal loops. TMM and ERL1 interact via their extracellular domains. The formation of the TMM-ERL1 complex increases the binding affinity of EPF1 and EPF2 to the receptor, because the N-terminal loop of TMM contacts with the two terminal loops of the ligands. In chapter 2, I tested whether our protein structures were accurate. I treated *Arabidopsis* seedlings with EPF1 ligands that had ERL1- or TMM-interacting residues mutated. My results show that EPF1 ligands harboring those mutations displayed reduced function. I mutated glutamate 379 on TMM to arginine. This mutation disrupted the interaction between TMM and ERL1 in vitro (Lin et al. 2017). I found this mutation impaired the function of TMM in vivo. Taken together, my data support our structural study. My work presented in chapter 2 also provide evidence showing that EPFL9 suppressed the negative regulation of EPF1 on stomatal formation by competing with EPF1 for receptor binding.

In plants, membrane-localized receptor-like kinases (RLKs) play important roles in diverse biological processes by sensing a wide range of external signals. RLKs containing extracellular leucine-rich repeats (LRRs) constitute the largest subfamily of RLKs, with about 240 members in *Arabidopsis* (Gou et al. 2010). So far, a small number of LRR-RLKs have been functionally characterized (Wu et al. 2016), and only few of them have ligands paired and been studied from the protein structure point of view (Song et al. 2017). Our structural study on ERfs, TMM, and EPF/ EPFL ligands might be able to provide a template for studying interactions of other LRR-RLKs and their ligands, which form similar structures as ERfs and EPF/ EPFL ligands.

The LRR is a structural motif that contains 20-30 amino acids with conserved leucines. It is found in a variety of organisms, including bacteria, fungi, plants and animals (Kobe and Kajava 2001). LRR domains are built from tandems of two or more LRRs. Animal LRR domains form horse-shoe shaped structures (Bella et al. 2008). However, our work on ERfs and research from other groups on brassinosteroid-insensitive 1 (BRI1), flagellin-

insensitive 2 (FLS2), receptor-like protein kinase 2 (RPK2) reveal that plant LRR domains adopt right-handed superhelical structures (Lin et al. 2017; Hothorn et al. 2011; Sun et al. 2013; Song et al. 2014). Protein sequence analysis shows plant LRR consensus sequences include a short sequence of GxIP (x stands for any amino acid), which does not exist in animal LRR consensus sequences (Torii 2004; Bella et al. 2008). It is hypothesized that this GxIP sequence is likely responsible for the formation of superhelical structures of plant LRR domains (Han et al. 2014). Whether this non-canonical structure has any biological meaning is not known. TMM interacts with ERL1 in a way that the N-terminal loop of TMM can help anchoring EPF1 onto ERL1 (Lin et al. 2017) (Figure 4.1A). If the LRR domain of ERL1 adopts a canonical horse-shoe shape, TMM has to reposition itself on ERL1, which might disrupt the contacts between TMM and EPF1. Flagelin22 (flg22) lays on the concave side of FLS2 in an extended and straight form (Sun et al. 2013) (Figure 4.1B). If the LRR domain of FLS2 is in a horse-shoe shape, flg22 has to bend in order to interact with the concave face of FLS2. Thus, a possible scenario is that the superhelical structures of plant LRR domains might mediate plant-specific receptor-coreceptor-ligand interactions. An intriguing question will be why plants evolve this unique structure for their LRR domains? Having a better understanding of how plant and animal LRR proteins function in the extracellular space may help answering this question.

ER exhibited weak kinase activity (Meng et al. 2015). It is unknown how ERf signal is transduced from the receptors. Based on our structural study and research from Meng and colleagues, we hypothesize that SOMATIC EMBRYOGENESIS RECEPTOR KINASE family receptors (SERKs) might act as partner proteins of ERfs in transducing ERf signal. When the structures of EPF1-ERL1^{LRR}-TMM^{LRR} and TDIF-PXY^{LRR}-SERK2^{LRR} were aligned, the hairpin loop of EPF1 pointed to SERK2 and might be able to interact with SERK2 (Lin et al. 2017). Protein pull-down assay revealed that ER and ERL1 interacted with SERKs in a ligand dependent manner (Meng et al. 2015). In vitro kinase assay showed that the intracellular domains of ER and SERK3 trans-phosphorylated each other (Meng et al. 2015). Additionally, the *serk1-1 serk2-1 serk3-5* displayed stomatal clustering phenotype similar to the *er erl1 erl2* mutant (Meng et al. 2015). These results indicate that ERfs and SERKs function together in stomatal regulation. To test our hypothesis, we would need to investigate if the loop regions

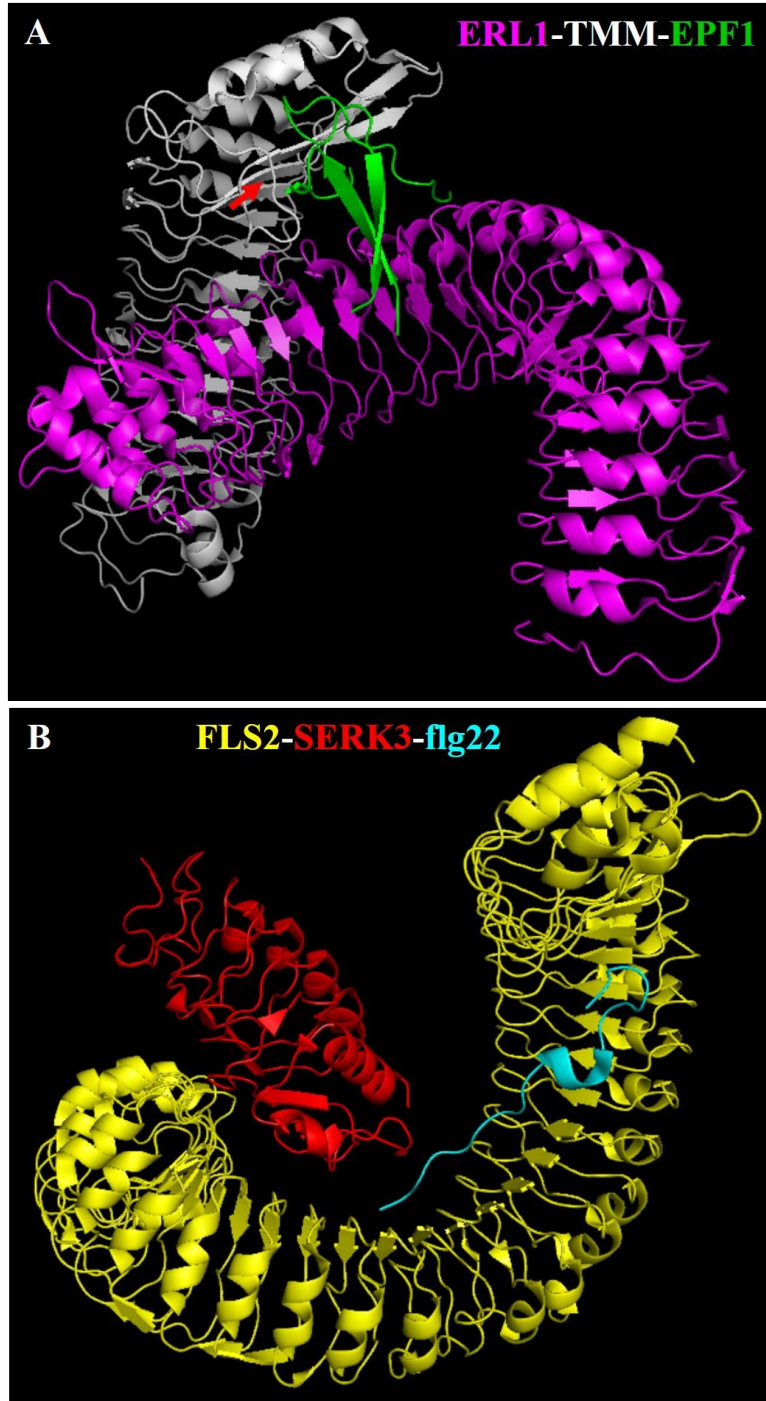


Figure 4.1 Protein structures of the ERL1-TMM-EPF1 and the FLS2-SERK3-flg22 complexes. (A and B) Shown are the LRR domains of ERL1 (magenta), TMM (grey), FLS2 (yellow), and SERK3 (red) and the ligands EPF1 (green) and flg22 (cyan). (A) Protein structure of the ERL1-TMM-EPF1 complex. PDB ID: 5XJO (Lin et al. 2017). Red arrow points to the N-terminal loop of TMM. (B) Protein structure of the FLS2-SERK3-flg22 complex. PDB ID: 4MN8 (Sun et al. 2013).

of EPF/ EPFL ligands interact with SERKs and recruit SERKs to ERfs. In our structural work, we did not include the intracellular domains of ERfs. However, to fully understand how ERfs function, we should also gain insight of their intracellular domains. We cannot exclude the possibility that ligand-binding on the extracellular domains of ERfs induces conformational changes of the intracellular domains. These changes activate ERfs and enable them to transduce signals.

Ligand binding to BRI1 or FLS2 results in heterodimerization of these two proteins with SERK3, which causes trans-phosphorylation of BRI1-SERK3 and trans-phosphorylation of FLS2-SERK3, and subsequent downstream signaling that controls plant growth and immune responses, respectively (Li et al. 2002; Nam and Li 2002; Chinchilla et al. 2007; Heese et al. 2007). An intriguing question raised here is that if ERfs, BRI1, and FLS2 all require SERKs for transducing signals, how can plants tell which external signal they should respond to? The signal specificity seems not to be achieved by differential expression, since ERfs, BRI1, and FLS2 exhibit spatiotemporally overlapping expression patterns (Shpak et al. 2005; Robatzek et al. 2006; Friedrichsen et al. 2000). A possibility could be that SERKs activate ERfs, BRI1, and FLS2, but signal transduction is mediated by the receptors themselves, which interact with different downstream signaling components, rather than by SERKs (Belkhadir and Jaillais 2015; Wan et al. 2019). However, it is worth noting that signal specificity could be determined by many factors, such as receptor amount on plasma membrane, the formation of plasma subdomains enriched in specific receptors and their partner proteins, the structure of extracellular matrix, the duration of stimulus, expression of scaffolding proteins, and interplay among different signaling pathways (Murphy and Blenis 2006; Lajoie et al. 2009). It would be interesting to know why SERKs are involved in ERf signaling. As signaling partners of ERfs, BRI1, and FLS2, SERKs might play an important role in modulating the tradeoff between different signaling pathways. Studying how SERKs influence different signaling pathways can help us gain a better understanding of how plants coordinate their growth under different conditions.

Our structural work and research from Abrash and colleagues suggest that EPFL4 and EPFL6, which are expressed in stem endodermis, may move to stem epidermis and suppress

stomatal formation in there (Lin et al. 2017; Abrash et al, 2011). EPFL4 and EPFL6 promote stem elongation (Abrash et al, 2011). It is unclear why these two ligands do not also promote stomatal formation, so that the efficiency of photosynthesis would be increased, and stem elongation would be better supported. EPFL4 and EPFL6 suppress stomatal formation when TMM is absent (Abrash et al, 2011). TMM is only expressed in the epidermis where stomata form (Nadeau and Sack 2002). It is possible that during evolution EPF/ EPFL ligands developed new functions, and TMM was selected to help ERfs to identify ligands that regulate stomatal development from ligands that also have other functions. Our in vitro protein binding assay shows that TMM enhanced the binding affinity of EPF1 and EPF2 to ERfs, while impaired the binding of EPFL4 and EPFL6 to ERfs (Lin et al. 2017). The interactions of ERfs, TMM, and EPF/ EPFLs may represent a common mechanism that exists in different organisms and explains why receptors that have multiple ligands can distinguish their ligands and perform different functions. It would be interesting to investigate whether other receptors use a TMM-like mechanism for ligand binding. The interactions of ERfs, TMM, and EPF/ EPFLs may also provide us an example to study how evolution shapes the structure of organisms and the underlying regulatory mechanisms.

Stomata control the exchange of water vapor and carbon dioxide between plants and the atmosphere. Proper stomatal formation is crucial for plants to survive and plays an important role in global water and carbon cycles (Hetherington and Woodward 2003). Our work reports the protein structures of ERfs, the receptors that play crucial roles in regulating stomatal development. Based on our work, we may be able to design molecules that can mimic the binding of EPF1/ EPF2 and EPFL9 to ERfs. Those molecules can be applied to plant surface to manipulate stomatal formation temporarily or in a long term. This could be a new way to improve the drought resistance of crops, reduce water usage in agriculture, regulate the growth rate of crops, and enhance their productivity. By manipulating stomatal formation in plants, we might also be able to reduce the amount of carbon dioxide in the atmosphere.

Chapter 3

My study demonstrates how ERfs regulate the SAM. In chapter 3, my genetic analysis revealed that ERfs and CLV3 synergistically suppressed SAM expansion. The *clv3 er erl1 erl2* mutant displayed a much larger SAM than either the *er erl1 erl2* mutant or the *clv3* mutant. Cells in the *clv3 er erl1 erl2* SAM remained undifferentiated. No lateral organs or stems were generated. My work also revealed that *WUS* is epistatic to *ERfs*. The *wus er erl1 erl2* mutant was phenotypically indistinguishable from the *wus* mutant. Both mutants did not have a dome-like SAM, and periclinal cell divisions were found in the center of their shoot apices. The expression of *WUS* and *CLV3* was increased in the *er erl1 erl2* mutant but decreased when ERf signaling was enhanced by exogenous application of EPFL4 or EPFL6. This suggests that *WUS* and *CLV3* are gene targets of ERf signaling. Kosentka and colleagues reported that the ligands of ERfs were expressed in the SAM boundary (Kosentka et al. 2019). Based on my work and research from Kosentka and colleagues (Kosentka et al. 2019), I propose that ERf signaling regulates the SAM via laterally restricting *WUS* and *CLV3* expression to the center of the SAM.

The communication between the organization center (OC) and the central zone (CZ), which is mediated by the *WUS*-*CLV3* loop, is essential for the maintenance of the SAM. It is important to understand how this loop works. It is also important to know how the expression of *WUS* and *CLV3* is restricted to the OC and the CZ, since the establishment of proper communication relies on fine-regulated gene expression. Recent research demonstrates that cytokinin (CK) signaling and HAIRY MERISTEM (HAM) transcription factors function as positional cues for limiting *WUS* and *CLV3* expression to the OC and the CZ respectively in a longitudinal manner (Chickarmane et al. 2012; Zhou et al. 2015). It is intriguing whether the expression of these two genes is also laterally regulated. Zhao and colleagues reported that MONOPTEROS (MP), which was activated by auxin in the peripheral zone (PZ) of the SAM, up-regulated *CLV3* expression in the CZ (Zhao et al. 2010). Their work indicates *CLV3* expression is indeed affected by signals from the sides of the SAM. However, it was unclear if *WUS* was regulated in the same way. My work presented in chapter 3 contributes to our

understanding of the establishment of the lateral regulation on *WUS* and *CLV3* expression. There are several questions that remain and would be interesting to be addressed in the future.

EPFL1, EPFL2, EPFL4, and EPFL6 are the ligands of ERfs, which redundantly regulate the SAM. The *epfl1 epfl2 epfl4 epfl6* mutant has an enlarged SAM and reduced leaf initiation rate as the *er erl1 erl2* mutant, whereas the lower-order mutants display weak or no SAM defects (Kosentka et al. 2019). The question is why the SAM is regulated by four EPFL ligands? CLV1 and BARELY ANY MERISTEM 1 (BAM1) are receptors of CLV3 (Fletcher et al. 1999; DeYoung et al. 2006). When there is functional CLV1, *BAM1* expression is repressed. Whereas, in the *clv1* mutant, *BAM1* is expressed and BAM1 receptor replaces the role of CLV1 for sensing CLV3 (Nimchuk et al. 2015). The regulation of *BAM1* expression by CLV1 may reflect a mechanism that buffers fluctuations in CLV1 levels to ensure a robust regulation of the SAM. Do EPFL1, EPFL2, EPFL4, and EPFL6 cross-regulate the expression of each other? If so, these four ligands will provide another example showing how the robustness of the SAM regulation is achieved.

EPFL1, *EPFL2*, and *EPFL4* are expressed in the boundary region of the SAM (Kosentka et al. 2019). Why are they expressed there? In other words, which signals trigger the expression of them in the SAM boundary? The boundary region is a transition zone between the inner meristematic region and the outer differentiating region. Auxin is transported out from this region (Vernoux et al. 2010). Is it possible that auxin depletion causes the expression of *EPFL1*, *EPFL2*, and *EPFL4*? However, recent study of leaf teeth formation showed that auxin signaling repressed *EPFL2* expression (Tameshige et al. 2016). Will what happens in the SAM boundary be opposite to what happens in leaf teeth? Or, the expression of *EPFL1*, *EPFL2*, and *EPFL4* is regulated by other signals, such as CK or CUP-SHAPED COTYLEDON (CUC) proteins? Knowing how *EPFL1*, *EPFL2*, *EPFL4* and *EPFL6* expression is controlled would help us understand why ERf signaling is chosen to regulate *WUS* and *CLV3* expression and would also help us gain a better picture of how the SAM is maintained.

In terms of SAM regulation, we know little about the downstream signaling events in ERf pathway. Expression of constitutively active YODA in the *er erl1 erl2* mutant recovered

the SAM and leaf initiation defects (Chen et al. 2013). This indicates the MAPKKK YODA functions downstream of ERfs in the SAM. However, what are the targets of YODA remains unknown. In stomatal lineage cells, MKK4/5 and MPK3/6 function sequentially after YODA. Are these kinases also involved in SAM regulation? If so, this MAPK cascade might act as a conserved signaling component of ERF pathways in different processes. Recent research showed that CLV3 signal was transduced by MPK3/6 (Lee et al. 2019). Is that possible that ERF and CLV3 signal converge at MPK3/6? Answering these questions would help us know the molecular mechanism underlying the SAM regulation mediated by ERfs.

Cell-cell communication mediated by the interaction of extracellular signals and corresponding receptors on the plasma membrane plays an essential role in plant and animal development. Besides this mechanism, plants share another way of intercellular communication, via plasmodesmata, which facilitate the exchange of signal molecules between neighboring plant cells. These two types of cell-cell communication cooperate in the SAM to ensure stem cell maintenance. *WUS* and SHOOT MERISTEMLESS (*STM*) move from their generation sites to stem cells through plasmodesmata to promote stem cell proliferation (Daum et al. 2014; Kim et al. 2003). EPFL ligands and CLV3 move via the apoplast until binding with receptors to down-regulate *WUS* expression (Kosentka et al. 2019; Brand et al. 2000). It is intriguing to ask why cells in the SAM adopt two different ways for communication? Cell-cell signaling via the symplast enables a single cell to control physiological processes under simple and homogeneous conditions. Whereas, intercellular communication via the apoplast allows cells to process and integrate multiple signals to make a decision that fits plant growth better under complex conditions (Chivasa and Goodman 2019). Within the SAM, there are only few cells underneath the CZ that express *WUS* (Mayer et al. 1998). Stem cells are highly homogenous. For *WUS* expressing cells to regulate stem cell population, it would be more efficient to use the symplastic pathway. However, since *WUS* is crucial for the maintenance of the SAM, to regulate the population of *WUS* expressing cells, it would be more appropriate to analyze all sorts of internal and external signals, such as the size of stem cell population, number of lateral organs, temperature, light conditions, et al. My work in chapter 3 and research from Kosentka and colleagues (Kosentka et al. 2019) indicate ERF signal is a CLV3-independent apoplastic signal that regulates *WUS*

expression. Future directions would be to address what signal ERfs deliver to WUS expressing cells and what are the other apoplastic signals.

During the post-embryonic growth of plants, the SAM generates all aboveground organs. The SAM is essential for plant survival and productivity. The components that affect the WUS-CLV3 loop have been shown to have dramatic impacts on the productivity of both tomato and maize (Xu et al. 2015; Je et al. 2016). *ERf* and *EPF/EPFL* genes are conserved in land plants (Villagarcia et al. 2012; Takata et al. 2013). Crop yield could be improved by modifying genes in these two families to acquire larger SAM. Beside regulating the SAM, ERfs also have impacts on stem elongation and phyllotaxis, modifying ERf signaling in ornamental plants might enable to produce new commercial varieties.

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