EPFL Signals in the Boundary Region of the SAM Restrict Its Size and Promote Leaf Initiation[1][OPEN]

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The shoot apical meristem (SAM) enables the formation of new organs throughout the life of a plant. ERECTA family (ERf) receptors restrict SAM size and promote initiation of leaves while simultaneously supporting establishment of correct phyllotaxy. In the epidermis and during organ elongation ERf activity is regulated by a family of Epidermal Patterning Factor-Like (EPFL) secreted Cys-rich small proteins. Here we show that ERfs play a critical role in communication between the SAM leaf boundary and the central zone in Arabidopsis (*Arabidopsis thaliana*). Ectopic expression of *ERECTA* in the central zone using the *CLAVATA3* promoter is sufficient to restrict meristem size and promote leaf initiation. Genetic analysis demonstrated that four putative ligands: EPFL1, EPFL2, EPFL4, and EPFL6 function redundantly in the SAM. These genes are expressed at the SAM-leaf boundary and in the peripheral zone. Previously EPFL4 and EPFL6 have been linked with elongation of aboveground organs. Here we demonstrate that EPFL1 and EPFL2 promote organ elongation as well. In addition, we show that expression of *ERECTA* in the central zone of the SAM has a strong impact on elongation of internodes and pedicels and growth of leaves. These results suggest that ERfs can stimulate organ growth cell nonautonomously.

Cell-to-cell communications coordinate numerous processes during plant development. Plant cells use both small organic molecules and peptides as message carriers. Plasma membrane-localized receptor-like kinases sense the majority of peptides and some organic molecules and then activate appropriate developmental programs. The ability of a receptor to sense multiple signals and the variety of responses a signal may trigger enable the complexity and plasticity of developmental programs.

The ERECTA family (ERf) signaling pathway was initially linked to aboveground organ elongation (Torii et al., 1996). Since then it has become clear that ERf receptors also regulate numerous other developmental processes such as stomata formation, leaf initiation, shoot apical meristem (SAM) structure, and flower differentiation (Shpak, 2013). In Arabidopsis (*Arabidopsis thaliana*), the family consists of three genes: *ERECTA*, *ERECTA-LIKE1* (*ERL1*), and *ERL2* (Shpak et al., 2004). The contribution of an individual receptor to the regulation of a particular developmental response varies. For example, ERECTA is the primary receptor regulating organ elongation whereas ERL1 plays a leading role in the regulation of stomata spacing. In the SAM these receptors function redundantly with single and double mutants exhibiting extremely weak or no phenotypes (Chen et al., 2013). The activity of ERf receptors is regulated by a family of 11 secreted Cys-rich small proteins from the Epidermal Patterning Factor/EPF-like (EPF/EPFL) family (Shimada et al., 2011). Three proteins—EPF1, EPF2, and STOMAGEN (EPFL9)—regulate stomata development (Hara et al., 2007, 2009; Hunt and Gray, 2009; Hunt et al., 2010; Sugano et al., 2010). Based on the phenotypes of mutants and on the fact that EPF2 is able to induce phosphorylation of downstream signaling components, EPF1 and EPF2 are thought to activate the receptors (Hara et al., 2007, 2009; Hunt and Gray, 2009; Lee et al., 2015). STOMAGEN competes with EPF1 and EPF2 for binding to ERfs but is unable to activate the downstream cascade, and thus functions as an antagonist (Ohki et al., 2011; Lee et al., 2015). Two ligands, EPFL4 and EPFL6, stimulate aboveground organ elongation (Abrash et al., 2011; Uchida et al., 2012). Another ligand, EPFL2, has been shown to regulate the shape of leaf margins (Tameshige et al., 2016). The rice (*Oryza sativa*) ortholog of EPFL1 induces awn elongation (Bessho-Uehara et al., 2016). No function for the remaining four potential ligands has been established. Selection of which ligands can bind to ERf receptors on a surface of an individual cell depends on the presence of the coreceptor TOO MANY MOUTHS, which promotes

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binding of EPF1, EPF2, and STOMAGEN and inhibits binding of EPFL4 and EPFL6 (Lin et al., 2017). The binding of ligands to ERfs or to ERf/TOO MANY MOUTHS complexes does not cause significant conformational changes or induce homodimerization of ERfs (Lin et al., 2017). ERfs might function in a complex with receptor-like kinases of the SERK family, which could potentially assist ERfs in activation of downstream targets (Meng et al., 2015). A MAP kinase cascade consisting of YODA, MKK4/5/7/9, and MPK3/6 transmits the signal downstream of ERfs (Bergmann et al., 2004; Wang et al., 2007; Lampard et al., 2009, 2014; Meng et al., 2012). The cascade is regulated by MAP KINASE PHOSPHATASE1 (Tamnanloo et al., 2018). How the signal is transmitted from the receptors to the cascade is not known.

Here we focus on ERf signaling in the SAM, a small but complex structure that must tightly control the proliferation and differentiation of its constituent cells. The SAM contains three different regions: the central zone with a pool of undifferentiated, slowly dividing cells; the peripheral zone where leaf and flower primordia are initiated; and the underlying rib zone, which provides cells for internodes. As cells are continually transitioned from the central zone into the other two, cell-to-cell communications are essential to maintain a relatively constant number of stem cells. These communications are achieved through a negative feedback loop consisting of the receptor/ligand pair CLAVATA1 (CLV1)/CLAVATA3 (CLV3) and the transcription factor WUSCHEL (WUS) (Clark, 2001). Presumably, the rate of cell proliferation and differentiation in the peripheral zone and the rib zone is also tightly controlled to ensure a consistent rate of organ initiation and uniformity of size; however, how this is achieved is not known. In addition, leaves and flowers develop in a specific geometric pattern. In Arabidopsis the SAM forms leaves and flowers at 137.5° angles to each other, producing a spiral pattern of these organs around the stem. The formation of auxin maxima determines the position of organ primordia (Sluis and Hake, 2015). ERfs play a critical role in these processes—the vegetative SAM of *er erl1 erl2* is dramatically wider and has a much broader central zone exhibiting increased expression of WUS (Chen et al., 2013; Uchida et al., 2013), and leaf primordia are initiated at a significantly reduced rate with almost random divergence angles (Chen et al., 2013). The changes in leaf initiation in *er erl1 erl2* correlate with abnormal auxin distribution as determined by a DR5rev:GFP marker and decreased *PIN1* expression in the vasculature (Chen et al., 2013).

To gain insight into the function of ERfs in the SAM, we explored their pattern of expression and searched for ligands that are perceived by ERfs. While ERfs are endogenously expressed throughout the SAM, their expression in the central region by the CLV3 promoter is most efficient in rescuing the meristematic defects of *er erl1 erl2*, compared to expression in the peripheral zone by the *KANADI* (*KAN*) promoter. Interestingly, ERECTA expression under the CLV3 promotor is also able to rescue leaf size and stem elongation phenotypes, suggesting that those parameters might be controlled by ERfs indirectly from distant tissues. Based on the phenotype of the quadruple mutant, ERfs can sense four ligands in the SAM: EPFL1, EPFL2, EPFL4, and EPFL6. Two of these ligands (EPFL1 and EPFL2) are expressed in the boundary region in the embryo and in the vegetative SAM. EPFL4 and EPFL6 are expressed at the periphery of the vegetative SAM. EPFL expression on the periphery of the meristem is critical as the *epfl1 epfl2 epfl4 epfl6* mutant can be rescued by EPFL1 expressed under the *KAN* promoter but not *CLV3*. Our data suggest that ERfs coordinate development of the central zone and the peripheral regions of the SAM.

RESULTS

Expression of *ERECTA* in the Central Zone is Most Efficient in Regulating the SAM Size

Based on an in situ analysis and a reporter gene assay, *ERfs* are expressed broadly in the vegetative SAM and throughout forming leaf primordia (Yokoyama et al., 1998; Shpak et al., 2005; Uchida et al., 2013). A gene expression profile of the inflorescence SAM suggests similar expression of *ERfs* in the central zone, the peripheral zone, and in the organizing center with only *ERL1* being upregulated in the central zone (Yadav et al., 2009). In this experiment the zones were defined by *CLV3*, *FILAMENTOUS FLOWER*, and *WUS* expression, respectively. We were interested in how the meristematic expression of *ERfs* affects plant morphology and whether *ERECTA* expression in a specific zone is sufficient to rescue defects observed in the *er erl1 erl2* mutant. With this goal in mind, five different promoters were chosen. The *SHOOTMERISTEMLESS* (*STM*) promoter was used to express the gene throughout the SAM (Long et al., 1996). The *CLV3* and *WUS* promoters were used to drive *ERECTA* expression in the central zone and in the organizing center, respectively (Mayer et al., 1998; Fletcher et al., 1999). The *AINTEGUMENTA* (*ANT*) promoter was used to induce *ERECTA* expression in the peripheral zone and broadly in the forming leaf primordia (Elliott et al., 1996). We expected the *KAN* promoter to express *ERECTA* at the outer edges of the peripheral zone and on the abaxial side of leaf primordia (Kerstetter et al., 2001; Yadav et al., 2014). The *ERECTA* sequence placed behind the described promoters contained all 26 introns. Previous work suggested that endogenous *ERECTA* cis-regulatory elements are localized in the promoter (Yokoyama et al., 1998), with introns being essential for mRNA stability; mRNA produced by intronless *ERECTA* is degraded at the 3′ end (Karve et al., 2011). Thus it is unlikely that introns contributed to the pattern of expression in the generated transgenic lines.

To examine *ERECTA* expression in the generated transgenic lines, we performed in situ hybridization on 3-d-old seedlings (Fig. 1A). In the wild type, *ERECTA* was detected throughout the SAM and in leaf primordia, although the signal was very weak. In the *pCLV3*:

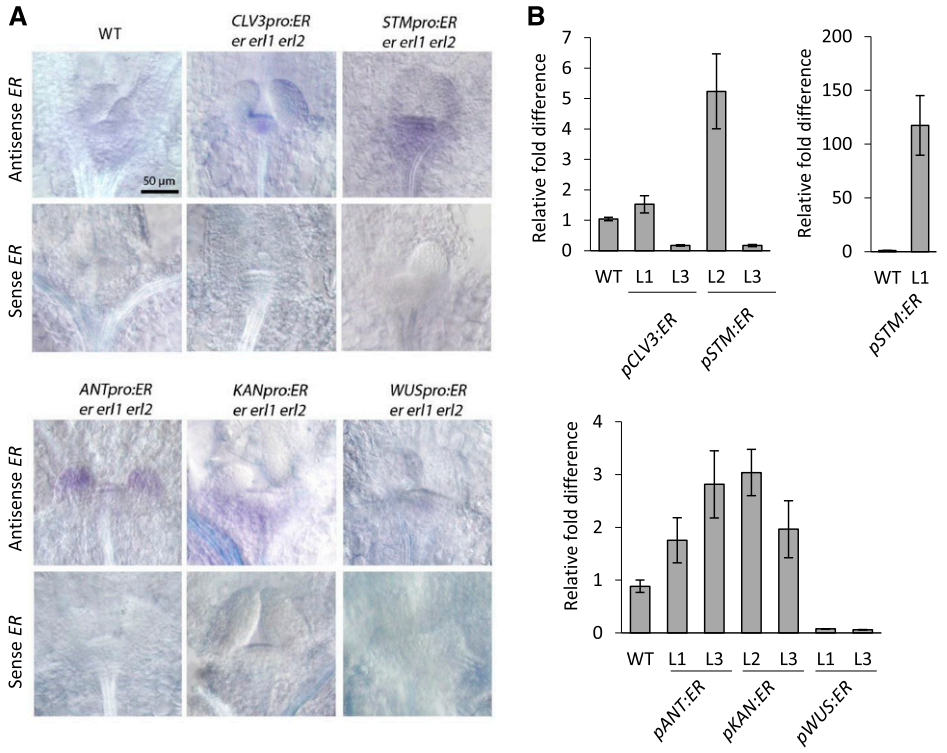

Figure 1. Ectopic expression of *ERECTA* (*ER*) in the SAM using heterologous promoters. A, Representative DIC images of in situ hybridization with a sense and an antisense probe for *ER* using 3-DPG T3 or T4 transgenic seedlings. B, RT-qPCR analysis of *ER* in 5-DPG seedlings of wild-type (WT) and transgenic plants. The average of three biological replicates is presented. Error bars represent SE. All images are under the same magnification.

ER and *pWUS*:*ER* transgenic plants, *ERECTA* was expressed as expected in the central zone and the organizing center, respectively. Based on both in situ and RT-qPCR, *ERECTA* expression was considerably lower in the *pWUS*:*ER* lines compared to all other transgenic lines (Fig. 1). Most importantly, in neither *pCLV3*:*ER* nor in p*WUS*:*ER* transgenic lines was *ERECTA* detected outside of the SAM. In *pSTM*:*ER* transgenic lines, a signal was observed throughout the SAM and sometimes on the abaxial side of leaf primordia. The strength of the in situ signal and its appearance outside of the meristem varied greatly, consistent with variable expression of *ERECTA* in those lines as determined by RT-qPCR (Fig. 1B). In the *pANT*:*ER* transgenic plants, in situ analysis detected *ERECTA* in the outer L1 layer of the SAM and throughout young organ primordia. A similar pattern, including expression in the L1 layer of the SAM, was observed previously when a 6.5-kb ANT promoter was used to drive *GUS* expression (An et al., 2004). In *pKAN*:*ER* transgenic plants, the majority of the signal was detected in the peripheral zone with very low expression in leaf primordia. Thus, the *CLV3* and *WUS* promoters drove expression of *ER* as expected, with the *STM*, *ANT*, and *KAN* promoters expressing *ERECTA* in slightly different patterns, suggesting that expression of genes under exogenous promoters should always be coupled with analysis of their expression.

To understand how zone-specific expression of *ERECTA* affects the SAM size, we analyzed transgenic seedlings 3 d post germination (DPG) and 5 DPG (Fig. 2). For each of the constructs, we have combined the data from three independent transgenic lines. Because we are interested in the differences between constructs and not between lines within a construct, the combined data improves the estimate of the mean parameter and reduces the SD of the mean, better resolving the differences between the constructs and controls. Because natural variation is expected to be present between lines, the net effect of combining the data is to broaden the overall distribution instead of narrowing it. Even with the additional broadening, the effect of the various constructs can be clearly seen in the raw data in Figure 2. The size of the meristem in lines expressing *ERECTA* throughout the meristem (under the *STM* promoter) or in the central zone (under the *CLV3* promoter) was rescued more efficiently compared to the other transgenic lines in both 3-DPG and 5- DPG samples (Fig. 2). The expression of *ERECTA* in the organizing center under the *WUS* promoter, in leaf primordia and the L1 layer of the SAM under the *ANT* promoter, and in the peripheral zone under the *KAN* promoter, were also able to rescue the SAM size but did so less efficiently (Fig. 2). This result cannot be attributed to the low expression of *ERECTA* in *pANT*:*ER* and *pKAN*:*ER* lines as determined by both RT-qPCR and in situ (Fig. 1, A and B). The reduced ability of *pWUS*:*ER* to rescue SAM size is probably only partially due to the low expression of *ERECTA* in those lines as low expression of *ERECTA* in *pCLV3*:*ER* line #3 and *pSTM*:*ER* lines #3 is sufficient to rescue the SAM size (Figs. 1B and 2). Thus, ERECTA can affect the meristem size when expressed in a variety of tissues, but it is most efficient when expressed in the central zone.

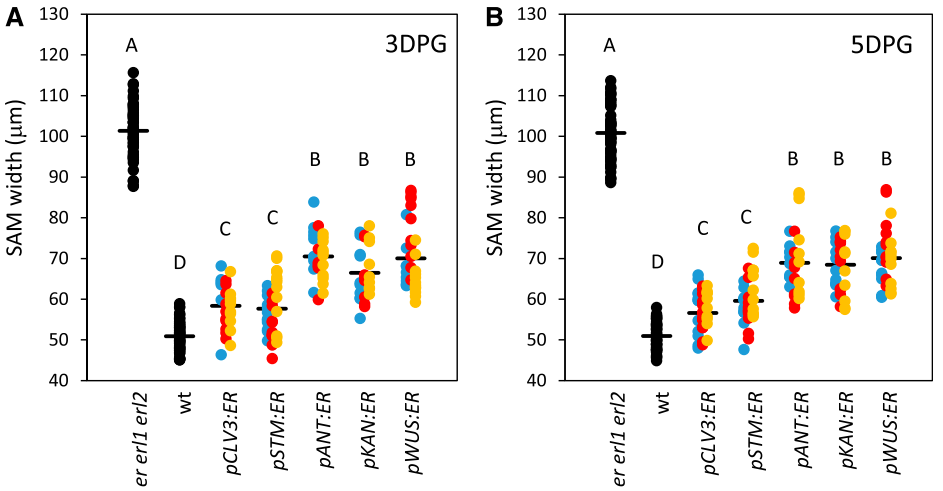

Figure 2. Expression of *ERECTA* in the central zone (*pCLV3:ER*) or broadly in the meristem (*pSTM:ER*) rescues SAM size defects the most effectively. SAM width measurements were performed by DIC microscopy using 3-DPG (A) and 5-DPG (B) seedlings. The width of SAM was measured as indicated by an arrow in Figure 8C. Three genetically independent transgenic lines were analyzed (line #1 is blue; line #2 is red; line #3 is yellow; *n* for each transgenic line = 7 to 11). The mean is indicated as a thick horizontal line. In all cases presented, the SD of the mean was less than 1.5 mm and can be considered insignificant. Different letters indicate significant difference at $P < 0.05$, as determined by one-way ANOVA with Tukey's post test.

Expression of *ERECTA* in the Central Zone of the SAM is Most Efficient in Regulating Leaf Initiation

ERfs promote leaf initiation (Chen et al., 2013). At 3 DPG and 5 DPG, the *er erl1 erl2* mutant forms on average 0.33 ± 0.03 and 0.42 ± 0.03 times as many leaves compared to the wild type. Out of the five promoters used, *CLV3* and *STM* were the most efficient in rescuing leaf initiation defects (Fig. 3). Plants expressing the *pCLV3:ER* and *pSTM:ER* constructs in the *er erl1 erl2* background formed very similar numbers of leaves at 3 DPG and an indistinguishable number of leaves at 5 DPG compared to the wild type. The *pANT:ER* transgenic plants had on average 0.85 ± 0.05 and 0.80 ± 0.06 times as many leaves compared to the wild type at 3 d and 5 d, respectively. It is not clear whether expression in the leaf primordia or in the L1 layer of the meristem is responsible for this phenotype. Expression of *ERECTA* in the peripheral zone using the *KAN* promoter or in the organizing center using the *WUS* promoter were the least efficient in enhancement of leaf initiation (Fig. 3). It is interesting to note that expression of *ERECTA* in the organizing center has an effect at all on leaf initiation in the peripheral zone, suggesting that at least to some extent ERfs regulate leaf initiation indirectly. Based on the phenotypes of *pCLV3:ER*, p*KAN:ER*, and *pWUS:ER* transgenic plants, we conclude that ERfs can regulate leaf initiation indirectly and they do so the most efficiently when expressed in the central zone of the meristem.

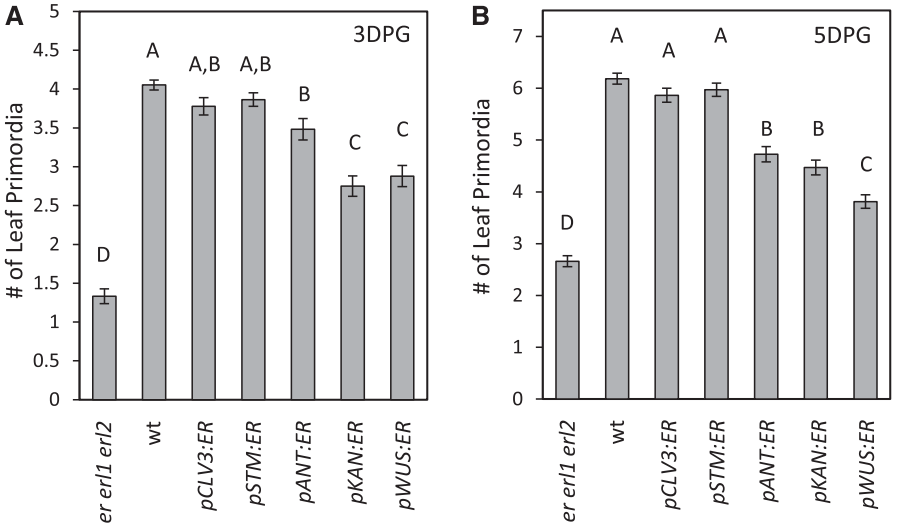

Figure 3. Expression of *ERECTA* in the central zone (*pCLV3:ER*) or broadly in the meristem (*pSTM:ER*) rescues leaf initiation most efficiently. The number of leaf primordia formed was measured by DIC microscopy using 3-DPG (A) and 5-DPG (B) seedlings. Three genetically independent transgenic lines were analyzed for each construct and the data were combined to determine the mean (*n* for each transgenic line = 7 to 11; total *n* for each construct = 27 to 65). Error bars represent SE. Different letters indicate significant difference at $P < 0.05$, as determined by one-way ANOVA with Tukey's post test.

ERECTA Expression in the SAM Can Alter Leaf Expansion and Stem Elongation

In addition to SAM size and leaf initiation, the expression of *ERECTA* under the utilized promoters altered other aspects of plant development. Two out of the five constructs, *pSTM*:*ER* and *pANT*:*ER*, were able to rescue infertility of *er erl1 erl2* (Supplemental Fig. S1), consistent with their expression during both early and late stages of flower development (Elliott et al., 1996; Long et al., 1996). *CLV3* and *WUS* are expressed only during early stages of flower development (Mayer et al., 1998; Fletcher et al., 1999), and thus it is not surprising that *ERECTA* expressed under promoters of those genes cannot rescue fertility defects. Although the *KAN* promoter is active on the abaxial side of initiating floral organs and in the tissue that gives rise to ovules (Kerstetter et al., 2001), that expression was not sufficient to rescue infertility of *er erl1 erl2* (Supplemental Fig. S1A).

The *ERf* genes are not only important for leaf initiation but also for leaf expansion (Shpak et al., 2004). In the three independent transgenic lines analyzed, the *STM* promoter led to very different levels of *ERECTA* transcription from approximately 100 to 150 times more than the wild type in line #1 to approximately five times less in line #3 (Fig. 1B). The expression was observed both in young primordia (Fig. 1A) and in mature leaves (Fig. 4B). The different levels of *ERECTA* expression were reflected in the size and shape of leaves, with fully rescued leaf expansion in line #1 and a minor increase in leaf expansion in line #3 (Supplemental Fig. S1B). Two *pCLV3*:*ER* lines also varied in the levels of *ERECTA* expression. The *pCLV3*:*ER* line #1 expressed approximately 1.5 more *ERECTA* compared to the wild type, and line #3 about six times less (Fig. 1B). This difference of expression again was reflected in different leaf sizes (Supplemental Fig. S1B). Comparison of line #3 *pSTM*:*ER* and line #3 *pCLV3*:*ER*, which on the level of the whole seedling express similar amounts of *ERECTA*, suggests that expression under the *CLV3* promoter is more efficient in promoting leaf expansion (Fig. 1B and Supplemental Fig. S1B). Interestingly, *ERECTA* expression directly in leaves using the *KAN* and *ANT* promoters only weakly altered leaf size (Fig. 4). The leaf size in *pKAN*:*ER* line #2, which had twice as much *ERECTA* in mature leaves compared to the wild type, was very similar to the size of leaves in *pWUS*:*ER* line #3 where *ERECTA* was barely detectable if even present. The most revealing line is *pANT*:*ER* line #1, which expressed relatively high levels of *ERECTA* throughout both young primordia and older leaves but only partially rescued leaf size. Taken together, these data

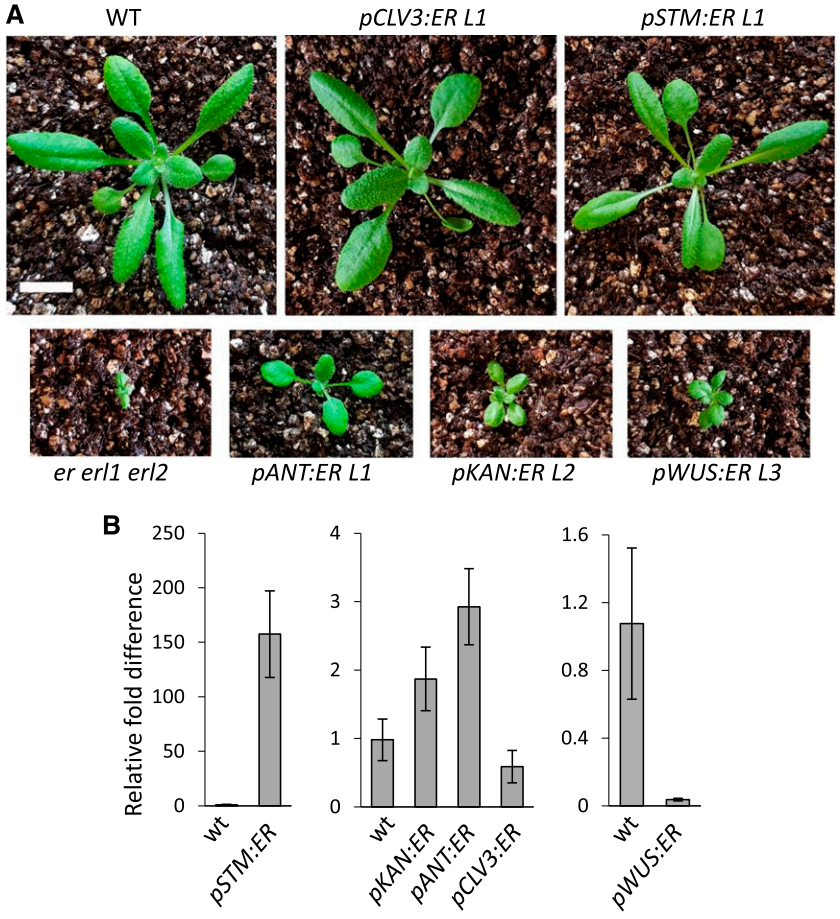

Figure 4. Expression of *ERECTA* using the *CLV3* or *STM* promoters most efficiently rescues leaf shape defects of the *er erl1 erl2* mutant. A, 20-DPG plants, bar = 1 cm. B, RT-qPCR analysis of *ER* in leaves of wild-type (wt) and T3 to T6 transgenic plants. The average of three biological replicates is presented. Error bars represent SE. All images are under the same magnification.

suggest that ERfs can regulate leaf size indirectly from the SAM.

To further explore how ectopic expression of *ERECTA* affects plant growth, we analyzed plant height and pedicel lengths. Previously it was shown that *ERECTA* expression in the phloem using the *SUC2* promoter was able to rescue height and pedicel length in the *erecta* mutant (Uchida et al., 2012). Here we show that expression in a variety of tissues rescues elongation defects of *er erl1 erl2* (Fig. 5). *ERECTA* most efficiently stimulated stem growth when expressed under *CLV3* and *STM* promoters, which is most noticeable when one observes younger plants (Supplemental Fig. S1C). Given enough time, *ERECTA* under the control of four promoters, *CLV3*, *STM*, *ANT* and *KAN*, fully rescued final plant height (Fig. 5A). *ERECTA* under the same four promoters also stimulated pedicel elongation in *er erl1 erl2* (Fig. 5B), with *pCLV3:ER* and *pKAN:ER* being the least efficient. This may be at least partially related to their inability to rescue fertility, because we previously demonstrated that pedicels attached to unfertilized siliques are approximately 2 mm shorter compared to those attached to fertilized siliques (Bundy et al., 2012). Unexpectedly, even low expression of *ERECTA* in the organizing center of the SAM using the *WUS* promoter had a small but statistically significant effect on both stem and pedicel elongation (Fig. 5; Supplemental Fig. S1C). These results suggest that expression of *ERECTA* in the phloem is not obligatory for regulation of organ elongation and *ERECTA* expressed in other tissues can promote organ elongation as well.

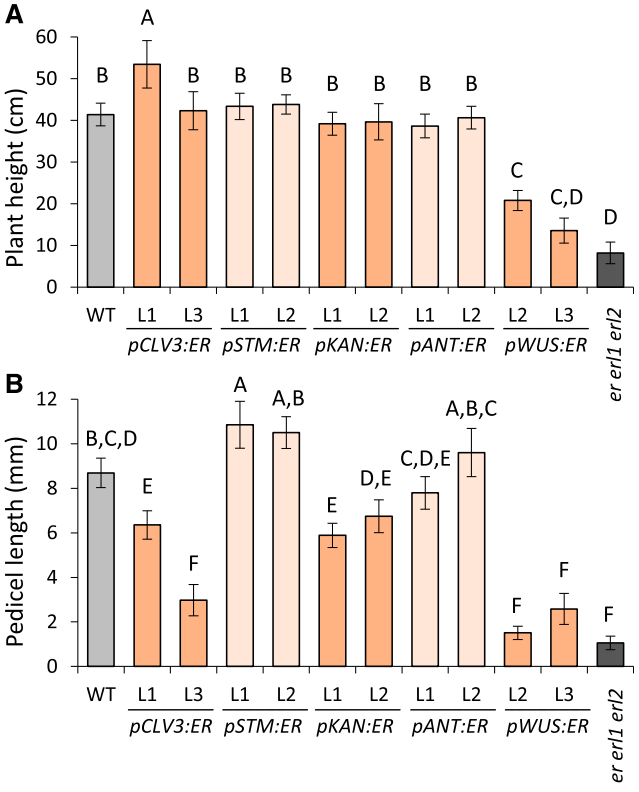

Figure 5. Expression of *ERECTA* under a variety of promoters can fully or partially rescue elongation of stem and pedicels in the *er erl1 erl2* mutant. Plant height (A) and pedicel length (B) were measured in mature 2-month-old plants. Two independent transgenic lines were analyzed; $n = 10$ to 30 for heights and $n = 64$ for pedicel length. Error bars represent SD. Different letters indicate significant difference at $P < 0.01$, as determined by one-way ANOVA with Tukey's post test.

Expression Pattern of *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* Near the SAM

The activity of ERf receptors is regulated by a group of secreted small proteins from the EPF/EPFL family (Shimada et al., 2011). To narrow down the group of ligands that might be perceived by ERfs in the SAM, we investigated *EPF/EPFL* expression patterns using the GUS and GFP transcriptional reporter assays. Analysis of whole mount seedlings using the GUS assay suggested that *EPF1* and *EPF2* are expressed in epidermis, and specifically in developing stomata, but not in the SAM (Supplemental Fig. S2). We were unable to detect expression of *EPFL8* at that developmental stage. The expression of other genes near the meristematic region was further examined by sectioning (Fig. 6A). Three genes, *EPFL3*, *EPFL5/CHALLAH-LIKE1*, and *EPFL7*, were expressed in different regions of leaf primordia: *EPFL3* on the adaxial side of leaves at some distance from the SAM; *EPFL5* at the base of leaf primordia, especially on the abaxial size; and *EPFL7* in the internal tissues at the base of leaf primordia. Four genes were clearly expressed near the meristematic region: *EPFL1*, *EPFL2*, *EPFL4/CLL2*, and *EPFL9/STOMAGEN*. We observed expression of *EPFL1* at the boundary and in L1 of the SAM and on the adaxial side of forming leaf primordia. *EPFL2* exhibited extremely strong expression at the boundary. *EPFL4* was expressed in the L3 layer of the peripheral zone. *EPFL9* was expressed in the rib zone of the meristem. Because *EPFL9* is an antagonist of ERfs (Lee et al., 2015) and currently mutants in that gene are unavailable, we did not investigate it any further. *EPFL6/CHAL* was not expressed directly in the SAM but its expression was detected in the inner tissues underneath leaf primordia peripheral to the SAM. Next, we used epifluorescence microscopy to analyze *EPFL* expression during embryogenesis. Out of 11 genes, *EPFL1*, *EPFL2*, and *EPFL6* were expressed in the developing embryos. *EPFL1* and *EPFL2* were expressed very highly in the peripheral regions of the embryonic SAM where margins of cotyledons meet (Fig. 6B). *EPFL1* was also expressed in the epidermis of the hypocotyl and in the root apical meristem. We detected expression of *EPFL6* in hypocotyl only during late embryogenesis. It was expressed in two cell files that are presumably endodermis.

EPFL1, *EPFL2*, *EPFL4*, and *EPFL6* Partially Redundantly Regulate Elongation of Plant Organs

Due to their expression near the meristematic region, we investigated the function of *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* in plant development. The *epfl4/cll2-1* and *epfl6/chal-2* single mutants are null alleles carrying

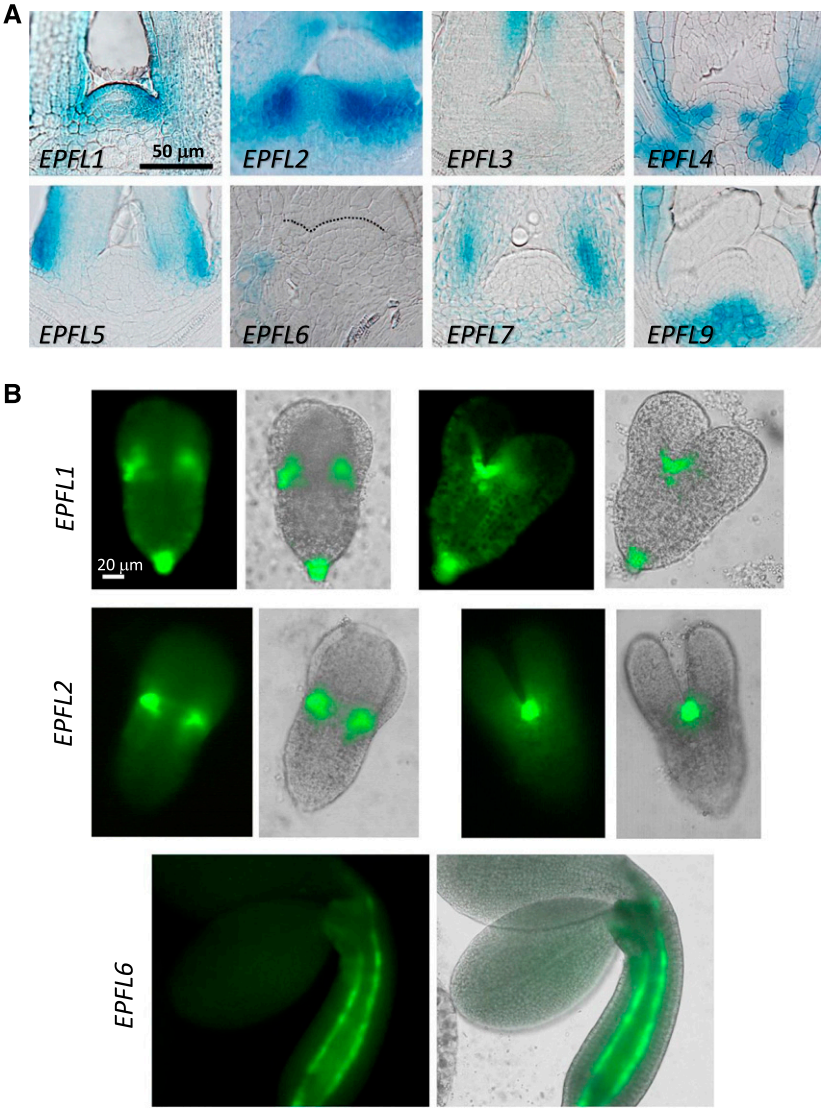

Figure 6. A reporter gene assay of the *EPF/EPFL* gene family in the SAM demonstrates distinct patterns of expression. A, Longitudinal sections of shoot apices of T2 7-DPG or 10-DPG wild-type seedlings expressing indicated *pEPFL:EGFP-GUS* constructs. The dotted line in the *EPFL6* insert emphasizes the L1 layer of the SAM. B, Epi-fluorescence microscopy of plants expressing *pEPFL1:EGFP-GUS* and *pEPFL2:EGFP-GUS* in torpedo embryos and *pEPFL6:EGFP-GUS* in bend cotyledons embryos. For the first two constructs, the same embryo is represented from two different perspectives. All images are under the same magnification in (A) and in (B).

T-DNA insertions with no visible phenotype (Abrash and Bergmann, 2010; Abrash et al., 2011; Uchida et al., 2012). The *epfl2-1* allele is a null allele carrying a transposon insertion and the mutant exhibits diminished leaf tooth growth (Tameshige et al., 2016). The *epfl1-1* allele is a null allele carrying a transposon insertion and the mutant has no visible phenotype (Supplemental Figs. S3 and S4). To understand the function of these genes we created all possible combinations of double and triple mutants. The *epfl4 epfl6* plants are shorter in stature compared to the wild type but are slightly taller compared to *er-105* (Fig. 7, A and B; Abrash et al., 2011; Uchida et al., 2012). None of the other double mutants displayed a significant reduction in elongation of stems or pedicels (Fig. 7, A and B). Addition of the *epfl1* mutation to *epfl4 epfl6* did not change stem and pedicel elongation, whereas the presence of *epfl2* in the *epfl4 epfl6* background slightly reduced elongation of pedicels, leading to formation of more compact inflorescence (Fig. 7, B and E). The *epfl1 epfl2 epfl4 epfl6* mutant reached a final height comparable to that of the *erecta* single mutant; however, it grew drastically slower and took an additional four weeks to achieve maturity compared to *erecta* (Fig. 7, A and D). In this respect *epfl1 epfl2 epfl4 epfl6* is similar to *er erl1 erl2*, which is also characterized by an extended period of growth and a longer lifespan (Kosentka et al., 2017). We observed that the extended life span of *epfl1 epfl2 epfl4 epfl6* leads to increased number of siliques formed on the main stem (Fig. 7C). Taken together, these data suggest that although *EPFL4* and *EPFL6* play the primary role in stimulation of stem and pedicel elongation, *EPFL1* and *EPFL2* also contribute to this process.

In addition to changes in elongation of aboveground organs we also observed changes in silique growth, fertility, and apical dominance (Supplemental Fig. S4.) Of all double mutants, *epfl1 epfl6* formed the shortest siliques, suggesting the primary role for these two genes is in fruit development (Supplemental Fig. S4B). Fertility was reduced in the *epfl1 epfl2 epfl6* and *epfl1 epfl4 epfl6* mutants, and *epfl1 epfl2 epfl4 epfl6* plants are

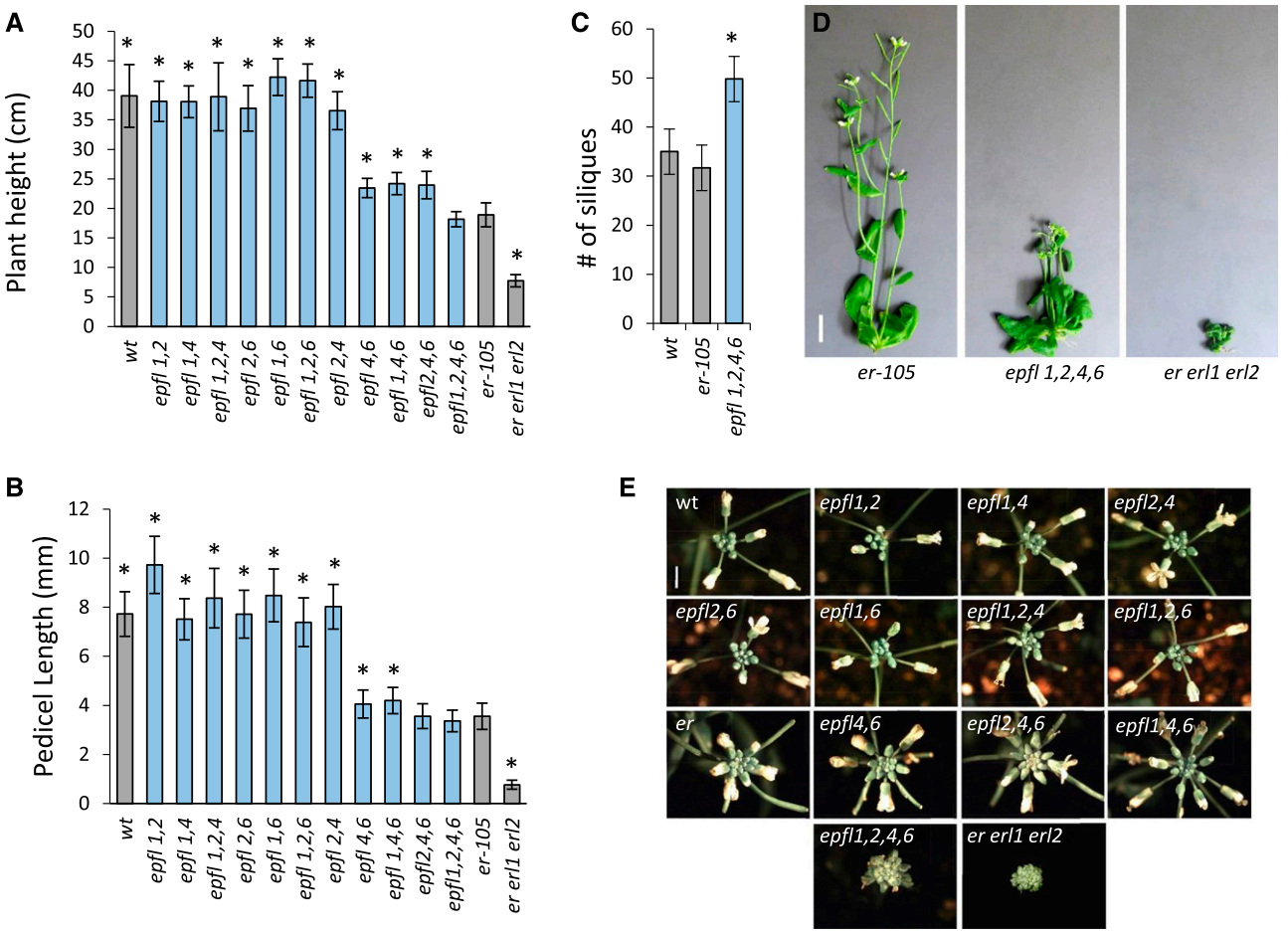

Figure 7. *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* synergistically regulate stem and pedicel elongation with *EPFL4* and *EPFL6* playing the key role. A, Height of fully grown plants ($n = 27$ to 46 except *er erl1 erl2*, $n = 12$). B, Lengths of mature pedicels on the main stem ($n = 100$ to 120). C, Number of siliques on the main stem ($n = 10$). A to C, Bars represent the average; error bars represent SD. Values significantly different from *er-105* are indicated by asterisks (based on Student *t* test; $P < 0.001$). D, 6-week-old plants of *er-105*, *epfl1-1 epfl2-1 epfl4 epfl6*, and *er-105 erl1-2 erl2-1*. Scale bar: 1 cm. E, Influorescence apices from the wild type, *er*, *er erl1 erl2*, and various combinations of *epfl* mutants. Bar = 25 mm. All images are under the same magnification in (D) and in (E). wt, wild type.

infertile (Supplemental Fig. S4A). In addition, all four genes contribute partially redundantly to establishment of apical dominance (Supplemental Fig. S4C). No obvious changes in the formation of stomata were observed (Supplemental Fig. S5).

EPFL1, *EPFL2*, *EPFL4*, and *EPFL6* Redundantly Regulate SAM Size and Leaf Initiation

Analysis of triple *epfl* mutants demonstrated a slight but statistically significant increase of meristem size in triple mutant combinations: *epfl1 epfl2 epfl4*; *epfl1 epfl2 epfl6*; and *epfl1 epfl4 epfl6* (Fig. 8A). There were no significant changes in the rate of leaf initiation (Fig. 8B). Because the *epfl1 epfl2 epfl4 epfl6* mutant is infertile and the epidermal phenotype cannot be used to identify it in the progeny of *erfl1/+ epfl2 epfl4 epfl6* plants, 30 seedlings with slightly shorter petioles of cotyledons were genotyped for *epfl1-1* before fixation for differential interference contrast (DIC) microscopy. This allowed us to identify 10 *epfl1 epfl2 epfl4 epfl6* mutants. The following analysis demonstrated that in terms of meristem size and leaf initiation rate *epfl1 epfl2 epfl4 epfl6* is indistinguishable from *er erl1 erl2* (Fig. 8), suggesting that the four genes *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* regulate meristem function. To confirm that the phenotype is due to mutations in the *EPFL* genes and not to some other overlooked mutations, we independently expressed *EPFL1* and *EPFL2* under their endogenous promoters in *epfl1 epfl2 epfl4 epfl6*. Both constructs rescued meristematic defects (Fig. 9), promoted stem and pedicel elongation (Supplemental Fig. S6), and rescued fertility defects in multiple independent transgenic lines. To test whether ligands have to be coexpressed with ERfs in the central zone or if they function from the peripheral zone, *EPFL1* was expressed in *epfl1 epfl2 epfl4 epfl6* under *CLV3* and *KAN* promoters. The expression under *KAN* fully rescued both meristem size and leaf initiation, whereas

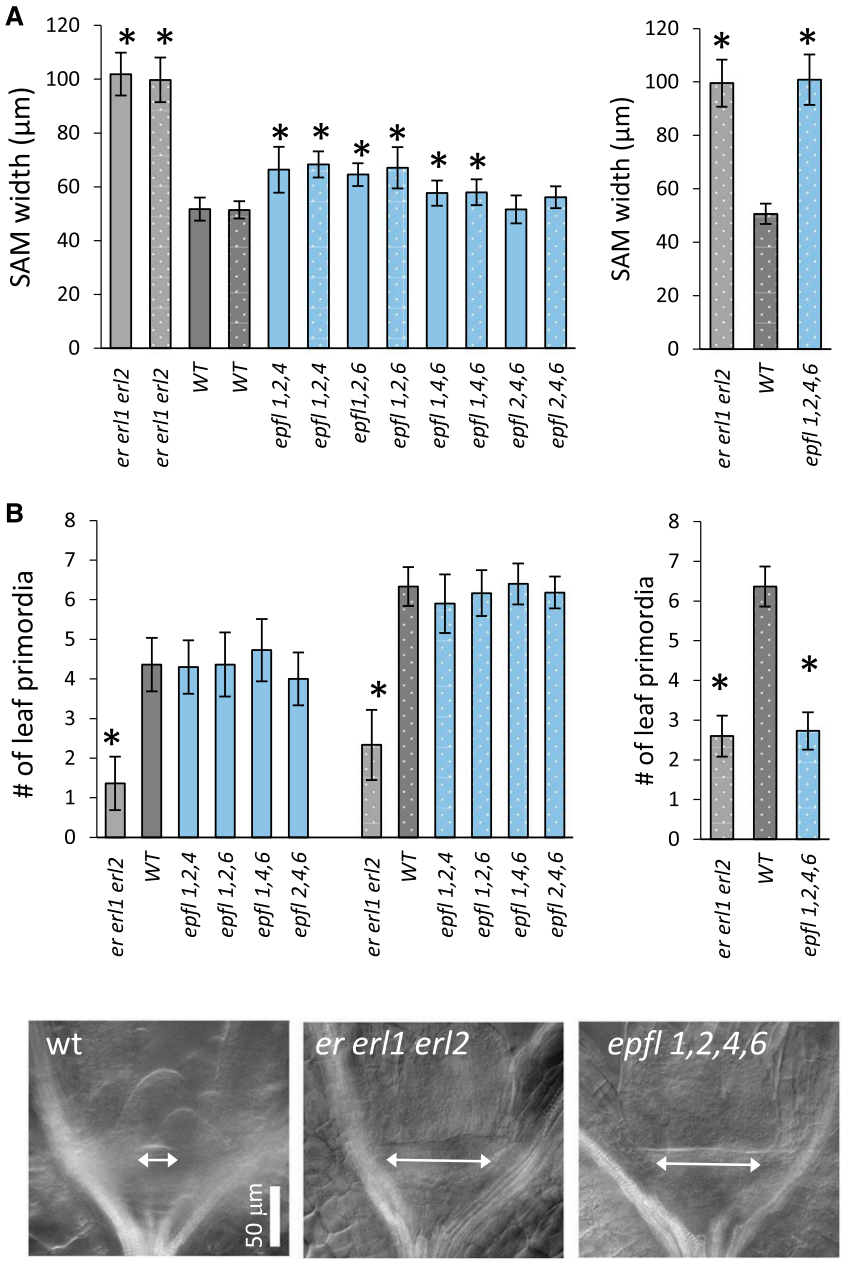

Figure 8. *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* redundantly regulate the size of the SAM and the rate of leaf initiation. Comparison of the SAM width (A) and the number of formed leaf primordia (B) in the wild type, *er erl1 erl2*, and *epfl* family mutants determined by DIC microscopy at 3 DPG (solid bars) and 5 DPG (dotted bars). Bars represent the average; error bars represent SD. $n = 10$ to 11. Values significantly different from the wild type are indicated by asterisks (based on Student *t* test; $P < 0.006$). C, DIC images of meristematic regions in the wild type, *er erl1 erl2*, and *epfl1,2,4,6* at 3 DPG. The meristem width is displayed with an arrow. All images are under the same magnification in (C). wt, wild type.

expression under *CLV3* had no effect on the meristem size and only partially rescued leaf initiation (Fig. 9). In addition, expression under *KAN* but not the *CLV3* promoter rescued organ elongation defects (Supplemental Fig. S6). Taken together, these data suggest that four *EPFL* genes, *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6*, redundantly regulate maintenance of meristem size and promote leaf initiation with expression in the peripheral zone being sufficient for their function.

DISCUSSION

The ERf signaling pathway first appeared in early land plants and has evolved to regulate multiple aspects of plant development (Villagarcia et al., 2012; Shpak, 2013; Takata et al., 2013). Whereas a species typically contains only two to three ERf receptors, the EPF/EPFL family of putative ligands is relatively large, with 10 or more genes being typical (Takata et al., 2013; Zhang et al., 2018). Each individual *EPF/EPFL* is expressed in a unique spatio-temporal pattern and often controls a specific developmental process. For example, in Arabidopsis, rice and the moss *Physcomitrella patens EPF1* orthologs control stomata development (Hara et al., 2007, 2009; Hunt and Gray, 2009; Caine et al., 2016, 2018).

The first indication that ERECTA signaling might contribute to regulation of SAM structure came from the analysis of higher order mutants. It was observed that the *er* mutation enhances meristematic defects

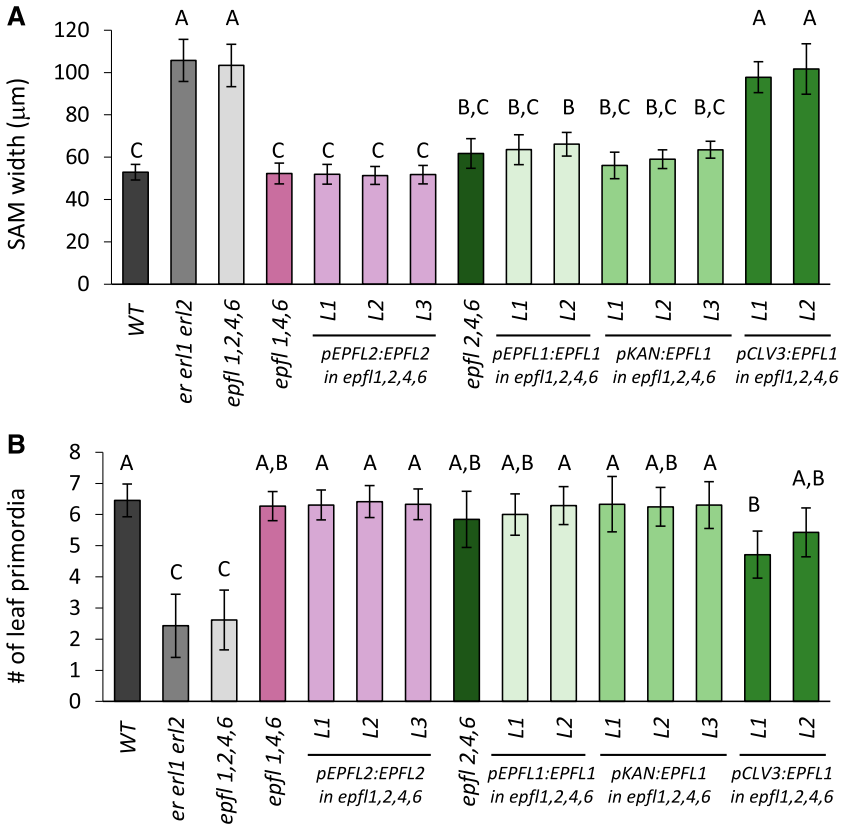

Figure 9. The meristematic phenotype of *epfl1,2,4,6* can be fully rescued by expression of *EPFL1* or *EPFL2* under endogenous promoters or by expression of *EPFL1* under *KAN* promoter but not *CLV3*. Comparison of the SAM width (A) and the number of formed leaf primordia (B) in the wild type (WT), selected mutants as indicated and in independent transgenic lines expressing indicated constructs in *epfl1,2,4,6* background as determined by DIC microscopy in 5-DPG seedlings. Bars represent the average; error bars represent SD. $n = 7$ to 14. Different letters indicate significant difference at $P < 0.01$, as determined by one-way ANOVA with Tukey's post test.

of CLV pathway mutants and suppresses those of the *uni-1D*/+ mutant (Diévart et al., 2003; Durbak and Tax, 2011; Uchida et al., 2011). Later, analysis of the *er erl1 erl2* mutant demonstrated that ERfs synergistically inhibit expansion of the vegetative meristem and promote leaf initiation (Chen et al., 2013; Uchida et al., 2013). While the CLAVATA pathway regulates meristem height, ERECTA signaling restricts the meristem width and functions independently of CLAVATA (Mandel et al., 2014, 2016). Understanding a signaling pathway depends on knowing the identity of cells involved in sending and receiving the signal. ERf receptors are expressed throughout the SAM and in forming leaf primordia (Yokoyama et al., 1998; Uchida et al., 2013), but that does not mean that their expression in all those areas is necessary for regulation of meristem expansion and/or leaf initiation. To uncover the regions where ERfs are critical for meristem maintenance and organ initiation, we expressed ERECTA under a range of promoters in the *er erl1 erl2* mutant. Unexpectedly, expression of ERECTA under all five promoters, *STM*, *CLV3*, *KAN*, *ANT*, and *WUS*, in different and in some cases nonoverlapping areas of the meristem reduced meristem size and promoted leaf initiation, suggesting that ERfs can have an impact on meristem function when expressed in a variety of locations. Simultaneously, expression of *ERECTA* throughout the meristem under the *STM* promoter or in the central zone under the *CLV3* promoter had the strongest impact on the meristem width and organ initiation, implying that the function of ERfs in the central zone is paramount. It is interesting to note that expression of *ERECTA* under the *WUS* promoter elements is insufficient to fully rescue meristematic defects of *er erl1 erl2* while expression of *CLV1* under the same promoter elements fully rescues the *clv1* mutant (Nimchuk et al., 2015), which reinforces the distinctiveness of these two signaling pathways.

The next question is: What signals are perceived by ERfs in the SAM? There are 11 EPF/EPFLs in Arabidopsis. Analysis of mutants suggests that four genes, *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6*, contribute to meristem size establishment and promotion of leaf initiation. These genes function redundantly with triple mutants exhibiting no or very weak meristematic phenotypes. *EPFL1*, *EPFL2* and *EPFL4*, *EPFL6* belong to two closely related clades with the stomata-regulating *EPF1*, *EPF2*, and *EPFL9* genes being more distantly related (Takata et al., 2013). Both clades have one additional gene, *EPFL3* and *EPFL8*, respectively, which are neither expressed near the meristematic region nor seem to be essential for SAM regulation. EPFL4 and EPFL6 are verified ERf ligands as they bind directly to ERfs (Lee et al., 2012; Lin et al., 2017). EPFL2 has been shown to bind to Erf-containing complexes, which suggests that genes belonging to that clade are likely to encode ERf ligands (Tameshige et al., 2016). Because all four genes have the potential to suppress stomata development when expressed in the epidermal tissue layer, they are likely to be agonists of ERf receptors

(Abrash et al., 2011). *EPFL1* and *EPFL2* are expressed during embryogenesis in the boundary region between two cotyledons at the periphery of the SAM. After germination, they are expressed in the analogous region at the border of the meristem and previously formed leaf primordia. Expression of *EPFL1* in the border zone is consistent with gene expression profiling of the inflorescence SAM, which indicated upregulated *EPFL1* expression at the periphery of the SAM (Yadav et al., 2009). *EPFL2* was classified as a boundary-enriched gene in a TRAP-seq experiment that was done using 7-d-old seedlings (Tian et al., 2014). The boundary zone has a low rate of cell divisions, low auxin accumulation, and high expression of CUC genes, which is similar to another location where *EPFL2* is expressed—the sinus of leaf teeth (Tameshige et al., 2016; Wang et al., 2016). *EPFL4* and *EPFL6* are expressed near the SAM after germination with *EPFL4* in the internal layers of the peripheral zone and *EPFL6* in the border region at some distance from SAM.

ERf expression in the central zone and the expression of *EPFLs* at the periphery of the meristem or at the bases of leaf primordia suggest that the ERf signaling pathway enables communications between the border region and the central zone. This conclusion is also supported by the ability of *EPFL1* to rescue the quadruple mutant phenotype when expressed under the *KAN* but not the *CLV3* promoter. Taken together, our data suggest that overlap in the expression of *EPFLs* and *ERfs* expression at the outer boundary of the central zone of the meristem restricts SAM width and promotes leaf initiation.

Recently it has been proposed that ERfs function in the L1 layer of the meristem where they sense signals coming from internal layers of the SAM (Kimura et al., 2018). Our data are inconsistent with this conclusion. Kimura et al. utilized only two promoters to interrogate the function of ERfs and did not measure the meristematic parameters at multiple developmental points using numerous samples to obtain statistically significant data. Because the expression of *ERECTA* in many different regions of the meristem alters behavior of meristematic cells, it is important to obtain quantitative measurements for precise comparisons. Moreover, it is necessary to take into account the differences in the expression levels of *ERECTA*. For example, while *ERECTA* expressed under the *ANT*, *KAN*, and *WUS* promoters rescues meristem defects in a similar manner, the first two promoters drive *ERECTA* expression at much higher levels, suggesting that the SAM is much more sensitive to *ERECTA* that is localized in the organizing center. In addition, our data suggest that ligands are expressed endogenously at the boundary of the meristem and in the peripheral zone and not in the internal layers. *EPFL* expression in the internal layers driven by the *CLV3* promoter cannot efficiently rescue the meristematic defects of the *epfl1 epfl2 epfl4 epfl6* mutant. Although Kimura and colleagues state that EPFLs are secreted in the internal layers of the SAM, data supporting that conclusion is not provided in their paper.

Expression of a gene under an exogenous promoter is a popular approach to interrogate gene function in a specific tissue. This approach has been effective in revealing the function of ERfs. Here, we would like to emphasize some issues associated with this approach. First, to prove that a gene controls a particular process from a specific tissue, it is necessary to use a sizable range of exogenous promoters. Because expression of *ERECTA* in a variety of nonoverlapping tissues has an effect on meristematic processes and elongation of organs, we believe that the use of a limited number of promoters has been misleading (Uchida et al., 2012; Kimura et al., 2018). Second, the expression pattern of a gene under an exogenous promoter can differ from what is expected, and it is essential to evaluate the actual expression pattern. For example, while in situ data suggest that *ANT* is expressed in leaf and flower primordia (Elliott et al., 1996; Long and Barton, 2000), the commonly used 6.5-kb promoter of that gene also drives expression in the L1 layer of the meristem (An et al., 2004). Finally, some promoters can lead to a variety of expression levels and expression patterns. An example is the *STM* promoter. In situ data indicate that *STM* is expressed throughout the SAM and is downregulated in the forming organ primordia (Long et al., 1996; Long and Barton, 2000). However, in transcriptional reporter assays the *STM* promoter induced diverse expression patterns that differed from the endogenous; The reporters were expressed underneath the SAM in the cells of the hypocotyl, in the vascular cells of the leaf primordia, preferentially at the boundary of SAM, or in the peripheral region but not the central region of the SAM (Kim et al., 2003; Verkest et al., 2005; Landrein et al., 2015). Similarly, in our experiments we observed *STM* expression underneath the SAM and in leaf primordia. Moreover, expression levels between created transgenic lines varied more than 500-fold. Hypothetically, these differences in expression could be due to inconsistent epigenetic regulation of the *STM* promoter in new locations (Katz et al., 2004).

By expressing *ERECTA* in different regions of the SAM, we anticipated the rescue of meristematic phenotypes. What we did not expect was to rescue the elongation of aboveground organs. *ERf* genes promote elongation of internodes, pedicels, petioles, siliques, leaves, and flower organs (Torii et al., 1996; Shpak et al., 2004). Previously it has been proposed that ERfs promote internode and pedicel growth by enabling cell-to-cell communication between the endodermis and phloem (Uchida et al., 2012). Our data suggest that *ERECTA* can promote organ elongation when expressed in a variety of locations including the central zone of the SAM. Most significantly, our data suggest that expression in the phloem is not essential for ERfs to promote elongation of organs. How can ERfs regulate organ elongation from the SAM? We can envision several mechanisms. Internodes are initially formed through activity of the peripheral zone that generates progenitor cells for epidermis, cortex, and of the rib zone that

supplies cells for the central cylinder. As observed above, the activity of *ERECTA* in the central zone promotes initiation of leaves in the peripheral zone. Thus, it is not a big stretch to imagine that ERfs promote proliferation of cells surrounding forming leaf primordia. Alternatively, ERfs might regulate growth of internodes indirectly, for example through controlling homeostasis of hormones such as auxin or gibberellin. This latter possibility can account for the ERf's ability to regulate organ growth when expressed in a variety of tissues, including from the phloem and the SAM. Our data indicate that the understanding of ERf's role in organ elongation is incomplete, and requires further investigation.

The phenotype of the *epfl1 epfl2 epfl4 epfl6* mutant suggests that, in addition to regulation of meristem structure, all four genes promote elongation of internodes and pedicels, with *EPFL4* and *EPFL6* playing the major role in this process. The expression pattern of *EPFL1* and *EPFL2* in internodes and pedicels and their precise role in organ elongation is yet to be established. Although the quadruple mutant grows much more slowly than *epfl4 epfl6*, its final size is only slightly below that of the *er* mutant and is considerably bigger compared to *er erl1 erl2*. This result suggests that either other ligands contribute to regulation of organ elongation or perhaps ERf regulates organ elongation by two mechanisms: in response to ligand binding and independently of ligand binding. Previously we demonstrated that the kinase dead ERECTA partially rescues organ elongation when expressed in *er erl1 erl2* (Kosentka et al., 2017). If the main outcome of EPF/EPFL binding is the activation of the ERf kinase domain, then it would be expected that the phenotype of *epfl1 epfl2 epfl4 epfl6* would resemble that of the kinase dead receptor, favoring the second hypothesis of two different mechanisms of ERf function in organ elongation.

MATERIALS AND METHODS

Generation of Transgenic Plants

Four different promoters (*STM*, *ANT*, *KAN*, and *WUS*) were independently cloned into pPZP222 vectors that carried the genomic *ERECTA* sequence and the endogenous 1.9 kb *ERECTA* terminator. The endogenous terminator does not have any regulatory sequences as all regulatory elements are localized in the *ERECTA* promoter (Yokoyama et al., 1998). In the constructs, the *35S* promoter drives expression of the selective marker gentamycin. To prevent this promoter from influencing expression of the transgenes, *ERECTA* and the selective marker were cloned in the head-to-tail orientation. Using the longer *ERECTA* terminator (1.9 kb) instead of the *35S* terminator (~200 bp) served the purpose of introducing spacer DNA between the two promoters to further reduce interactions between the transgene and the *35S* promoter. *pSTM:ER* (pPZK 311) was generated by amplifying a 4.62-kb region upstream of the *STM* start site. A similar 4.5-kb STM promoter region has been used and was analyzed in Verkest et al. (2005). *pWUS:ER* (pPZK 310) was created by amplifying a 4.5-kb region upstream of the *WUS* start site. This promoter region has been used previously by Yadav et al. (2009). *pANT:ER* (pPZK 315) was created by amplifying a 4.3-kb region upstream of the *ANT* start site as in Grandjean et al. (2004). *pKAN:ER* (pPZK 312) was generated by amplifying a 3.6-kb region upstream of the *KAN* start site as in Wu et al. (2008). The fifth construct *pCLV3:ER* (pPZK317) was generated slightly differently due to the presence of an enhancer in the terminator of *CLV3* (Brand et al., 2002). The genomic *ERECTA* sequence was inserted into pPZP222 between the 1.5-kb sequence upstream of the *CLV3* start site and the 1.2-kb sequence downstream of the *CLV3* stop codon. All promoter/ERECTA/terminator sequences were cloned into pPZP222 between *Bam*HI and *Xba*I restriction sites. All created constructs were examined by the restriction analysis and sequencing of amplified regions.

The described plasmids were transformed into an *Agrobacterium tumefaciens* strain GV3101/pMP90 by electroporation and introduced into *er erl1/+ erl2* plants by the floral dip method. The *er-105 erl1-2 erl2-1* mutant has been described in Shpak et al. (2004). The T1 transgenic plants were selected based on gentamicin resistance. Kanamycin resistance was used to identify *erl1-/+* or *erl1−/−* lines in the T2 generation. In the T3 or T4 generation, we selected lines that are homozygous for the transgene based on gentamicin resistance.

To generate *pEPFL1:EGFP-GUS*, a 1.5-kb fragment upstream of the *EPFL1* start site was PCR-amplified and inserted into p-ENTR/topo (Invitrogen) and recombined using LR recombinase (Invitrogen) into pKGWFS7 (Karimi et al., 2005). To clone the promoters of *EPF1* (*2.7 kb*), *EPF2* (*2.7 kb*), *EPFL2* (*3 kb*), *EPFL3* (*2.9 kb*), *EPFL4* (*2.8 kb*), *EPFL6* (*2.9 kb*), *EPFL7* (*1.5 kb*), *EPFL8* (*2.4 kb*), and *EPFL9* (*2 kb*) in front of *EGFP-GUS* a modified version of the Rapid one-step recombinational cloning method was used (Fu et al., 2008). The promoter regions were amplified by PCR using gene-specific primers that also contained shortened AttL1 or AttL2 sequences. Each fragment was extended using attL1-T2.1 and attL2-T2.1 primers to produce complete AttL sequences on both sides of each fragment. The generated fragments were recombined into pKGWFS7 using LR recombinase (Invitrogen). Primer sequences can be found in Supplemental Table S1.

The generated *pEPFL:EGFP-GUS* plasmids were introduced into wild-type plants as described above. The transgenic plants were selected based on kanamycin resistance. *pEPFL5:GUS* transgenic plants were described in Abrash and Bergmann (2010) and Abrash et al. (2011).

To generate *pEPFL1:EPFL1* a 3.3-kb fragment encompassing a 2-kb region upstream of the *EPFL1* start site and 0.8-kb region downstream of the stop codon was amplified and cloned into pPZP222. pEPFL2:EPFL2 was generated by amplifying a 4.2-kb fragment including 2.5 kb upstream of the *EPFL2* start codon and 1 kb downstream of the stop codon. In *pKAN:EPFL1* and *pCLV3:EPFL1* constructs, we used the same promoter regions as in *pKAN:ER* and *pCLV3:ER* and the *EPFL1* sequence that included introns. The *pKAN:EPFL1* construct contains the endogenous 0.8-kb *EPFL1* terminator whereas *pCLV3:EPFL1* contains the 1.2-kb sequence downstream of the *CLV3* stop codon.

In Situ Analysis

In situ hybridization was performed as described in Hejátko et al. (2006) using 3-DPG T3 and T4 transgenic or wild-type seedlings. One-kb cDNA region of *ERECTA* between the *Sac*I and *Xho*I restriction sites was cloned into pBluescript II and used as the template for in vitro transcription with T3 (Promega) and T7 (Invitrogen) RNA polymerases to make the sense and antisense probes, respectively. To generate *EPFL* probes, their full-length coding DNA sequences were amplified using wild-type cDNA and primers that contained the T7 promoter sequence near either the start or the stop codons. All probes were hydrolyzed to produce fragments with an average length of about 0.3 Kb. Representative images were taken using DIC microscopy.

Analysis of Mutant Phenotypes

For measurements of leaf number and SAM size by DIC microscopy, seedlings were grown on plates containing modified Murashige and Skoog medium supplemented with vitamins (Research Products International) and 1% (w/v) Suc. Selected 3-DPG and 5-DPG seedlings were incubated in a solution of 9:1 ethanol/acetic acid overnight, rehydrated using an ethanol series (90%, 80%, 70%, and 50%) and cleared in a chloral hydrate solution (chloral hydrate/water/glycerol 8:1:1). The *pSTM:ER* transgenic lines were analyzed in the T4 or T5 generations, as they were homozygous for the transgene and the *erl1* mutation. The p*WUS:ER*, *pKAN:ER*, *pANT:ER*, and *pCLV3:ERECTA* transgenic lines were analyzed in the T3 generation. These were homozygous for the transgene but were segregated for *erl1*. The *er erl1 erl2* plants used for analyses were identified based on the presence of stomata clusters in cotyledons. Microscopic observations were done using an Eclipse 80i microscope (Nikon) with DIC optics and NIS-Elements BR imaging software (Nikon) was used for measurements. For measurement of plant height and pedicel length and to observe leaf growth, plants were grown as described in Kosentka et al. (2017).

Generation of the *epfl* Double, Triple, and Quadruple Mutants

The *epfl1-1* (CS104435) transposon-insertion mutant (Columbia background) was obtained from the Arabidopsis Biological Resource Center. The *Epfl2-1*

(CSHL ET5721) transposon-insertion mutant (Landsberg *erecta* background) was received from Cold Spring Harbor Laboratory and outcrossed three times to *epfl1-1* to obtain *epfl1-1 epfl2-1* in the Columbia background. The absence of the *er-1* mutation in *epfl1-1 epfl2-1* was confirmed by sequencing. The *epfl1 epfl2* double mutant was crossed with *epfl4 epfl6 /cll2-1 chal-2* (Abrash et al., 2011) to obtain new combinations of mutations. *Epfl* double, triple, and quadruple mutants were identified by genotyping with *epfl1-1* and *epfl2-1* primers from Supplemental Table S2 and with *epfl4/cll2-1*, *epfl6/chal-2* specific primers described in Abrash et al. (2011). We used a three-primer PCR for genotyping of *epfl1-1* and *epfl2-1*. During genotyping of *epfl1-1*, the primers epfl1.436.rev and 3dspm were used to amplify an approximately 200-bp fragment and the primers epfl1.436.rev and epfl1.74 were used to amplify a 387-bp fragment. During genotyping of *epfl2-1*, the primers epfl2.1 and gus.43.rc were used to amplify an approximately 700-bp fragment and the primers epfl2.1 and epfl2.540.rev were used to amplify a 575-bp fragment. Because the *epfl1 epfl2 epfl4 epfl6* mutant is infertile, for the morphological analysis we obtained it from the progeny of *erfl1/+ epfl2 epfl4 epfl6* plants.

The GUS Reporter Gene and Assay and Microscopy

GUS staining was performed as described in Sessions et al. (1999) using 5-DPG T2 or T3 transgenic seedlings. Multiple independent transgenic lines were analyzed for each construct to find a consistent pattern of expression. Depending on the level of the signal, the concentration of ferricyanide and ferrocyanide in the staining buffer varied between 2 and 6 mM. After staining, the samples were dehydrated with a graded ethanol series up to 50% ethanol, fixed in FAA solution (3.7% formaldehyde/5% acetic acid/50% ethanol) for 30 min, dehydrated with a graded series of ethanol to 100% ethanol, infiltrated with polymethacryl resin Technovit 7100, and then embedded and polymerized in Technovit 7100 (Heraeus Kulzer). Eight-micrometer sections were prepared using a Leica RM-2255 microtome. Pictures were obtained using an Eclipse 80i microscope (Nikon) and a 12-megapixel cooled color DXM-1200c (Nikon) camera. A C-FL B-2A (Nikon) filter cube was used to observe the GFP signal.

Reverse Transcription PCR

Total RNA was isolated from five DPG seedlings and from fully expanded leaves using a Spectrum Plant Total RNA Kit (Sigma-Aldrich). RNA was treated with RQ1 DNase (Promega) and first-strand cDNA was synthesized using 150 ng of RNA with a ProtoScript II RT-PCR Kit (New England Biolabs) according to the manufacturer's instructions. Quantitative PCR was performed using the CFX96 Real Time System (BioRad) with Sso Evagreen Supermix reagent (BioRad). Each experiment used three technical replicates and three biological replicates to calculate relative fold difference of *ERECTA* to *ACTIN-2* expression. Bio-Rad CFX Manager was used to calculate cycle threshold values and the fold difference in gene expression was calculated using the delta-delta-Ct algorithm ($2^{-\Delta\Delta Ct}$). Primers and annealing temperatures are listed in Supplemental Table S3.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed here are as follows: ER (At2g26330), ERL1 (At5g62230), ERL2 (At5g07180), EPFL1 (At5g10310), EPFL2 (At4g37810), EPFL4 (At4g14723), and EPFL6 (At2g30370).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The effect of *ERECTA* expression under different promoters on plant morphology.

Supplemental Figure S2. The GUS reporter gene assay of the *EPF/EPFL* gene family.

Supplemental Figure S3. *Epfl1-1* is a null mutant with a transposon insertion in the second exon.

Supplemental Figure S4. *EPFL1*, *EPFL2*, *EPFL4*, and *EPFL6* partially redundantly regulate flower development and apical dominance.

Supplemental Figure S5. The *epfl1,2,4,6* mutant does not exhibit obvious stomata patterning defects.

Supplemental Figure S6. Expression of *EPFL1* under the endogenous and *KAN* promoters and *EPFL2* under endogenous promoter rescues elongation of stem and pedicels in the *epf 1,2,4,6*.

Supplemental Table S1. Primers used for cloning.

Supplemental Table S2. Primers used for genotyping *epfl1-1* and *epfl2-1*.

Supplemental Table S3. Primers used for RT-PCR.

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