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Stem development through vascular tissues: EPFL-ERECTA family signaling that bounces in and out of phloem

Authors/Affiliations:

Toshiaki Tameshige[1], Shuka Ikematsu[1,2], Keiko U Torii[1,2,3,4,†], Naoyuki Uchida[1,2,†]

[1] Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan

[2] Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602, Japan

[3] Department of Biology, University of Washington, Seattle, WA98195, USA

[4] Howard Hughes Medical Institute, University of Washington, Seattle, WA98195, USA

E-mail address ([†] Corresponding authors):

Toshiaki Tameshige; t.tame@itbm.nagoya-u.ac.jp

Shuka Ikematsu; ikematsu.shuka@f.mbox.nagoya-u.ac.jp

[†] Keiko U. Torii; E-mail: ktorii@u.washington.edu, Tel: +1-206-221-5701

[†] Naoyuki Uchida; E-mail: uchinao@itbm.nagoya-u.ac.jp, Tel: +81-52-789-2841

Highlight: This paper summarizes the history of ERECTA research including studies on stomatal development, then introduce ER functions in vascular tissues and discuss its interactions with phytohormones and other receptor pathways.

Running title: EPFL-ERECTA signaling that bounces in and out of phloem

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Abstract

Plant cells communicate with each other using a variety of signaling molecules. Recent studies have revealed that various types of secreted peptides, as well as phytohormones known since long ago, mediate cell-cell communication in diverse contexts of plant life. These peptides affect cellular activities, such as proliferation and cell fate decisions, through their perception by cell-surface receptors located on the plasma membrane of target cells. *ERECTA* (*ER*), an *Arabidopsis thaliana* receptor kinase gene, was first identified as a stem growth regulator, and since then an increasing number of studies have shown that ER is involved in a wide range of developmental and physiological processes. In particular, molecular functions of ER have been extensively studied in stomatal patterning. Furthermore, the importance of ER signaling in vascular tissues of inflorescence stems, especially at phloem cells, has recently been highlighted. In this review article, first we briefly summarize the history of ER research including studies on stomatal development, then introduce ER functions in vascular tissues, and discuss its interactions with phytohormones and other receptor-kinase signaling pathways. Future questions and challenges will also be addressed.

Key words: endodermis; EPFL; ERECTA; ligand; peptide; phloem; receptor; stem; stomata; vasculature

1. Brief early history of ERECTA research

The *erecta* (*er*) mutant of *Arabidopsis thaliana* was first isolated in 1950s as a single recessive mutant displaying a compact inflorescence (Rédei, 1992). The mutant was generated by x-ray radiation of Rédei's Landsberg seeds. This mutant line was thus called Landsberg *erecta* (L.*er*) and since then has been widely used as a "wild type" in a large number of *Arabidopsis* studies because of its space-saving compact stature for laboratory use. However, though it is believed that the parental Landsberg line was the La-1 accession (Zapata *et al.*, 2016), the authenticated parental line was unfortunately lost.

When the *ER* gene was cloned, known phenotypes were mostly limited to compact inflorescence with short internodes, short pedicels, blunt fruits, and also altered organ shape such as round leaves with short petioles (Rédei, 1962; Torii *et al.*, 1996). There were also interesting reports yet to be explained at molecular level. For instance, *er* mutation promotes stem growth in the *acaulis1* mutant background that displays extremely short stems (Tsukaya *et al.*, 1993), which is in contrast to the original *er* phenotype of compact stems. Later, a number of other phenotypes caused by attenuation of ER activity have been reported; leaf adaxial-abaxial polarity (Qi *et al.*, 2004; Xu *et al.*, 2003), pathogen response (Godiard *et al.*, 2003; Häffner *et al.*, 2014; Llorente *et al.*, 2005; Sánchez-Rodríguez *et al.*, 2009), transpiration efficiency (Masle *et al.*, 2005), flowering time (Hall *et al.*, 2007), shade avoidance (Patel *et al.*, 2013), drought tolerance (Shen *et al.*, 2015; Villagarcia *et al.*, 2012) and thermotolerance (Shen *et al.*, 2015).

Identification of two *Arabidopsis* genes closely related to *ER*, *ER-LIKE1* (*ERL1*) and *ERL2*, has further expanded *ER*-related research (Shpak *et al.*, 2004). These three genes compose *ER family* (*ERf*), and redundantly act for a variety of developmental processes. The triple mutant *er erl1 erl2* confers extreme dwarfism, enlarged shoot apical meristem (SAM), sterility and clustered stomata formation (Chen *et al.*, 2013; Hord *et al.*, 2008; Pillitteri *et al.*, 2007a; Shpak *et al.*, 2004; Shpak *et al.*, 2005; Uchida *et al.*, 2013). Increasing evidence highlights the involvement of ER in unusually diverse processes in plant development and physiology.

2. Ligands and other components acting for ER-family signaling pathway revealed

by stomata research

The *ER* gene encodes a receptor kinase consisting of an extracellular Leucine-Rich Repeat (LRR) domain, a single transmembrane domain and a cytosolic kinase domain (Torii *et al.*, 1996). Therefore, it was plausible to expect the existence of ligands that would bind to the LRR domain, and the activated ER proteins would in turn trigger downstream signaling. In addition, overexpression of a kinase-truncated version of ER exerted a dominant-negative effect that induced more severe phenotypes than the *er* single mutant, suggesting that ER would associate with interacting partners (Shpak *et al.*, 2003). Though such ligands and components in all ER-related processes reported have not fully unveiled yet, a number of factors have been identified in the past decade, especially from studies on stomatal patterning. Stomata development follows a characteristic cell division sequence (Fig. 1A), thus serving as a good model system to study developmental patterning.

The triple mutant *er erl1 erl2* forms clusters of stomata (Fig. 1B) (Shpak *et al.*, 2005). Such clusters are hardly observed in wild-type plants as stomata are usually formed with at least one non-stomatal cell between stomata according to the "one-cell-spacing rule" (Fig. 1B). This observation suggests that ERf and its hypothetical ligand(s) are involved in the cell-cell communication that prevents the formation of clustered stomata. Meanwhile, from a reverse-genetic screen in which 153 genes encoding small secreted peptides were overexpressed in wild type, EPIDERMAL PATTERNING FACTOR1 (EPF1) and EPF2 were identified as secreted peptides regulating stomata development (Hara *et al.*, 2007; Hara *et al.*, 2009; Hunt and Gray, 2009). Both EPF1 and EPF2 are closely-related cysteine-rich peptides and prevented stomata formation when overexpressed. Importantly, these effects depended on ERf, suggesting that EPF1 and EPF2 peptides might act as ligands which physically bind to ERf receptor kinases (Hara *et al.*, 2007; Hara *et al.*, 2009). This idea was later proved by biochemical experiments (Lee *et al.*, 2012). These studies also demonstrated that EPF2 peptides are primarily perceived by ER (Fig. 1C; EPF2-ER module) in an early step of stomata formation, preventing protodermal cells from entering stomatal lineage. On the other hand, EPF1 peptides mainly act on ERL1 (Fig. 1D; EPF1-ERL1 module) in a later step, inhibiting formation of stomata next to the existing one. In the *Arabidopsis* genome,

nine genes encoding EPF-LIKE (EPFL) peptides were identified, and the eleven factors including EPF1 and EPF2 compose EPF/EPFL family (Hara *et al.*, 2009). Of these gene products, EPFL9/STOMAGEN was shown to act as a positive regulator of stomata formation, behaving antagonistically to EPF2 and possibly also ERF1 (Fig. 1C, D) (Hunt *et al.*, 2010; Kondo *et al.*, 2010; Lee *et al.*, 2015; Sugano *et al.*, 2010).

TOO MANY MOUTHS (*TMM*) was identified as the gene responsible for enforcing the one-cell-spacing rule. (Nadeau and Sack, 2002; Yang and Sack, 1995). *TMM* encodes a receptor-like protein with an extracellular LRR domain and a single transmembrane domain but no kinase domain, implicating that TMM requires an interacting partner to trigger its downstream signaling. Interestingly, *tmm* mutant not only forms stomata clusters but also is insensitive to EPF2, like *er erl1 erl2* mutant (Hara *et al.*, 2009). Later, it was shown that TMM associates with ERf, acting as a co-receptor (Lee *et al.*, 2012). Recently, SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family receptor kinases, which act as co-receptors for many other LRR-type receptor kinases, including brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (Li *et al.*, 2002; Sun *et al.*, 2013a), bacterial peptide receptor FLAGELLIN-SENSITIVE 2 (Chinchilla *et al.*, 2007; Sun *et al.*, 2013b), abscission-regulating receptor HAESA (Meng *et al.*, 2016) and PHYTOSULFOKINE RECEPTOR (Santiago *et al.*, 2016; Wang *et al.*, 2015), was also shown to interact with ERf (Jordá *et al.*, 2016; Meng *et al.*, 2015). Accordingly, multiple mutant of *SERK* family genes exhibits stomata cluster phenotype (Meng *et al.*, 2015), showing that, in addition to TMM, SERK family proteins also function as co-receptors in ERf signaling (Fig. 1C, D).

ERf activates mitogen-activated protein (MAP) kinase cascade to regulate stomatal patterning. This cascade consists of YODA (YDA) as a MAP kinase kinase kinase, MKK4/5 as MAP kinase kinases and MPK3/6 as MAP kinases (Bergmann *et al.*, 2004; Lampard *et al.*, 2009; Wang *et al.*, 2007). The EPF2-ER signaling destabilizes the transcription factor SPEECHLESS (SPCH) that specifies the initiation and proliferation of stomatal lineage cells (Fig. 1C) (Jewaria *et al.*, 2013; Lampard *et al.*, 2008; MacAlister *et al.*, 2007; Pillitteri *et al.*, 2007b). On the other hand, the transcription factor that acts as a substrate of MPK3/6 downstream of the EPF1-ERL1 signaling remains unknown (Fig. 1D).

Thus, studies on stomatal patterning have led the identification and dissection of ligands and other components of ERf signaling (Fig. 1C, D). It will be important to investigate what parts of the framework drawn by stomata research are conserved in other ERf-related developmental and physiological processes and what parts are different. Below we summarize functions of ERf in vascular tissues and also discuss questions and challenges to be addressed in future.

3. Regulation of inflorescence architecture through endodermis-phloem communication

Loss-of-function *er* mutation confers compact inflorescence, pedicels, and siliques due to reduced cell numbers (Fig. 2A) (Torii *et al.*, 2003). Yet, the molecular nature of the ligand perceived by ER to regulate inflorescence architecture had long been unclear since *ER* gene was cloned in 1996 (Torii *et al.*, 1996). Identification of EPF1/EPF2/EPFL9 as the ligands for ERf in stomatal patterning led to the idea that a ligand(s) acting for ER-dependent inflorescence development might also be one or some of EPFL-family peptides.

EPFL-family genes are found in a wide range of land plant species, and the EPFL peptides are classified into four subgroups based on their amino acid sequences (Takata *et al.*, 2013). *EPF1, EPF2* and *EPFL7* are included in the same subgroup which is closely related to another subgroup including *EPFL9*, which is also known as *STOMAGEN* (Takata *et al.*, 2013). The remaining two subgroups are one including *EPFL1, EPFL2* and *EPFL3*, and the other including *EPFL4, EPFL5, EPFL6* and *EPFL8* (Takata *et al.*, 2013), though *EPFL8* may constitute an additional subgroup (Bessho-Uehara *et al.*, 2016).

Recent studies elucidated that EPFL4 (known also as CHALLAH-LIKE2 [CLL2]) and EPFL6 (CHALLAH [CHAL]) redundantly regulate inflorescence growth as *epfl4 epfl6* double mutant showed compact inflorescence strikingly similar to *er* mutant (Abrash *et al.*, 2011; Uchida *et al.*, 2012). Furthermore, it was shown that EPFL4/6 peptides physically interact with ER (Abrash *et al.*, 2011; Uchida *et al.*, 2012). Moreover, the MAP kinase cascade consisting of YDA, MKK4/5 and MPK3/6 acts as downstream signaling of ER in the inflorescence regulation as well as the stomata patterning (Meng *et*

al., 2012), suggesting that the common signaling module is adopted for these biological processes (Fig. 2C). Substrates of MPK3/6 in regulating inflorescence architecture are still unknown. Such substrates may be transcription factors like SPCH in stomatal development. It is noteworthy that dependence on TMM differs between EPF1/2 and EPFL4/6 (Abrash *et al.*, 2011). EPF1/2 require TMM to activate ERf, while EPFL4/6 do not. Rather, in the presence of TMM, EPFL4/6 do not efficiently stimulate the ER signaling. This apparently paradoxical situation implies that TMM, which is expressed in stomatal-lineage cells, functions to minimize a signal bleed through from internal tissues of stems, where *EPFL4/6*, but not *EPF1/2*, are expressed (see the following paragraph) (Abrash *et al.*, 2011; Torii, 2012). It is interesting to further note that *serk* family mutant resembles *er* inflorescence phenotypes (Meng *et al.*, 2015), although the requirement of SERKs for the EPFL4/6 perception by ER has not been tested.

Identification of EPFL4/6 and analysis of their expression patterns led a discovery of a novel cell-cell communication promoting inflorescence growth (Uchida *et al.*, 2012). In developing inflorescence stems, *EPFL4/6* are specifically expressed in endodermis (Fig. 2C). On the other hand, the *ER* expression is detected in phloem and xylem tissues as well as epidermis in stems. Since the specific expression of *ER* in phloem companion cells but neither of its xylem- nor epidermis-specific expression rescued the inflorescence phenotypes of *er*, ER activity in the phloem is the key to regulate the inflorescence architecture. Taken together, it is proposed that EPFL4/6 peptides secreted from endodermis are perceived by ER in phloem companion cells, and the activated ER signaling in the phloem cells promotes growth of inflorescence tissues (Fig. 2C).

In inflorescences of *er* mutants, cell numbers in cortex are reduced compared with those of wild type, while the mutant cells are elongated possibly due to compensatory cell growth (Fig. 2A) (Bundy *et al.*, 2012; Shpak *et al.*, 2004; Shpak *et al.*, 2003; Uchida *et al.*, 2012). Interestingly, this phenomenon was also rescued by the phloem-specific expression of *ER* (Uchida et al., 2012), suggesting that unknown secondary signal derived from the phloem affects cell proliferation in surrounding stem tissues (Fig. 2C). Given that phloem expresses groups of genes for specific metabolic pathways, some of which can affect stem growth (e.g. polyamine) (Kakehi *et al.*, 2008;

Pommerrenig *et al.*, 2011), such phloem-derived metabolites may mediate the inflorescence growth by ER.

Although some phytohormones promote stem elongation, relationships between ER signaling and such phytohormones largely remain to be understood. A suppressor screening of *er* mutant demonstrated that over-accumulation of auxin by *YUCCA5* overexpression compensates for the reduced growth of stems and pedicels in *er* (Woodward *et al.*, 2005). This compensation is not simply due to the rescue of cell proliferation in *er*, rather the enhancement of cell elongation. On the other hand, the effect of *YUCCA*5 overexpression on organ growth is enhanced by *er* mutant, suggesting that ER signaling buffers auxin action (Woodward *et al.*, 2005). Indeed, auxin response patterns are altered in the SAM and embryos in *er erl1 erl2* (Chen and Shpak, 2014; Chen *et al.*, 2013; Tameshige *et al.*, 2016). Very recently, it has been demonstrated that previously uncharacterized EPFL2 peptides play a role in shaping the auxin response pattern via ERf in leaf serration development (Tameshige *et al.*, 2016). Furthermore, the involvement of auxin in stomatal development has also been reported (Balcerowicz *et al.*, 2014; Le *et al.*, 2014; Zhang *et al.*, 2014). Collectively, accumulating evidence emphasizes coordinated action between auxin and ERf signaling.

Cytokinin (CK)-accumulating *ckx3 ckx5* mutant forms longer pedicels and fruits than wild type, which is opposite of *er* phenotypes (Bartrina *et al.*, 2011). On the other hand, *ckx3 ckx5* also shows thickened stem and enlarged SAM (Bartrina *et al.*, 2011), which are similar to phenotypes of *er* and *er erl1 erl2*, respectively (Torii *et al.*, 1996; Chen *et al.*, 2013; Uchida *et al.*, 2013). It was also reported that the SAM of *er erl1 erl2* exhibits enhanced responses to CK (Uchida *et al.*, 2013). Although these observations appear partly controversial, a possible hypothesis may be that the ER signaling buffers CK responses in both way; it may prevent CK response from diminishing and suppressing excess CK response depending on developmental contexts.

Gibberellin (GA) has long been known for its stem-elongating activity in many plants (Arney and Mancinelli, 1966; Sachs *et al.*, 1959). Consistently, a microarray analysis revealed that *er* and *epfl4 epfl6* mutants commonly show changes in expression of some GA metabolic genes (Uchida *et al.*, 2012). It was also reported that the short stem phenotype of the GA-insensitive mutant *short internode* (*shi*) is enhanced by the *er*

mutation (Fridborg *et al.*, 2001). Furthermore, the stem elongation phenotype of the GA-hyperresponsive mutant *spindly* (*spy*) is suppressed by *er* (Swain *et al.*, 2001). However, recent studies on *SHI* and *SPY* functions imply their involvement also in auxin biosynthesis and CK responsiveness, respectively (Steiner *et al.*, 2012; Baylis *et al.*, 2013). Therefore, ER might modify the *shi* and *spy* phenotypes through modulating auxin and/or CK actions.

It is still obscure how the ER signaling is integrated with phytohormone actions for stem growth. Yet, it would be intriguing to examine whether phytohormones mediate cell-cell signaling from phloem to cortex cells, in light of the non-cell autonomous nature of ER-mediated stem growth (Fig. 2C) (Uchida *et al.*, 2012). It would also be important to investigate which phytohormone could be metabolized in phloem upon the activation of ER signaling and which cell type responds to such a phytohormone.

4. Roles for EPFL-ER family signaling in vascular development

In the above section, we described that ER acts for stem elongation. In addition to *ER, ERL1* is also expressed in inflorescence stems (Abrash *et al.*, 2011; Uchida *et al.*, 2012), while *erl1* single mutant does not show an obvious stem phenotype (Shpak *et al.*, 2004). However, interestingly, the double mutant *er erl1* exhibits a defect in vascular tissues of stems. In *er erl1* stems, phloem cells are frequently located adjacent to xylem cells without intervening procambial cells, which is not observed in wild type, single *er* or *erl1* mutants (Fig. 2B) (Uchida and Tasaka, 2013). The phenotype of direct contact of xylem and phloem also occurs in a mutant of *TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR RECEPTOR/ PHLOEM INTERCALATED WITH XYLEM* (*TDR/PXY*), which encodes an LRR-type receptor kinase expressed in procambium (Fisher and Turner, 2007; Hirakawa *et al.*, 2008). Therefore, both ERf and TDR proteins function for procambial maintenance by promoting cell proliferation and/or suppressing cell differentiation. Genetic analyses suggested that these two pathways act in parallel as almost all procambial cells are consumed in *tdr er* double mutant stems, which is even more severe defect than *er erl1* and *tdr* phenotypes (Etchells *et al.*, 2013; Uchida and Tasaka, 2013). Like *ER*, the *ERL1* expression is also detected in epidermis, phloem and xylem (Uchida and Tasaka, 2013), and interestingly the procambium defect

in *er erl1* stems was rescued by phloem-specific activity of ER (Uchida and Tasaka, 2013) like the case of inflorescence phenotypes in *er* (Uchida *et al.*, 2012). The phloem-specific ER activity also recovered the severe phenotype of *tdr er* to the level of *tdr* single mutant. Therefore, the ER function in the phloem cells accounts for procambial maintenance independently of TDR. It is suggested that these two receptor systems differentially affect proliferation of procambial cells and their spatial differentiation pattern into xylem and phloem (Fig. 2D) (Etchells *et al.*, 2013). Interestingly, although *er tdr* shows more severe defects in procambial maintenance and vascular organization than *tdr* (Etchells *et al.*, 2013; Uchida and Tasaka, 2013), *tdr er* has similar numbers of vascular cells per bundle compared with *tdr* (Etchells *et al.*, 2013). This implicates that compensational cell proliferation occur in vascular bundles of *tdr er*.

ER and TDR receptors perceive their respective ligands. The ligand of TDR is TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) peptides encoded by *CLV3/ESR1-LIKE41* (*CLE41*) and *CLE44* (Ito *et al.*, 2006; Hirakawa *et al.*, 2008). TDIF is secreted from phloem and perceived by TDR at neighboring procambium (Fig. 3B) (Hirakawa *et al.*, 2008; Etchells and Turner, 2010). On the other hand, EPFL4 and EPFL6 act as ligands of ERf for procambial maintenance of stems as well as for stem elongation, as the severe vascular disorganization of *er tdr* was phenocopied by triple mutant *epfl4 epfl6 tdr* (Uchida and Tasaka, 2013). As mentioned above, EPFL4 and EPFL6 peptides are specifically produced in the endodermis (Uchida and Tasaka, 2013), indicating that the endodermis-phloem communication mediated by the EPFL4/6-ERf module plays an important role in vascular development (Fig. 2D). In summary, the two distinct ligand-receptor systems are required for procambial maintenance, which coordinately underlies proper tissue organization in vascular bundles (Fig. 2D) (Etchells *et al.*, 2013; Uchida and Tasaka, 2013).

It should be noted that, although *epfl4 epfl6* severely deteriorates the *tdr* phenotype like the *er* mutation, *epfl4 epfl6* does not show any obvious vascular phenotype by itself unlike *er erl1* (Uchida and Tasaka, 2013). This implicates the existence of an additional ligand(s) that activate the ERf signaling besides EPFL4/6 for vascular development in stems, leading the following two hypotheses (Fig. 2D). One is that EPFL4/6 and the additional ligand similarly activate both of ER and ERL1. The other

297 is that EPFL4/6 activate only ER just as the case for stem elongation, and the additional
298 ligand activates ERL1. The latter scenario is reminiscent of the stomatal regulation
299 primarily by the EPF2-ER module and the EPF1-ERL1 module (Fig. 1C, D) (Lee *et al.*,
300 2012). Both hypotheses can explain the genetic redundancy between *ER* and *ERL1* for
301 vascular phenotypes of *er erl1*.
302 Outputs of ER signaling activated in phloem need to be transmitted to
303 procambium to regulate its maintenance. Since there are several phytohormones involved
304 in vascular development and also in ER or TDR pathways, they might participate in the
305 phloem-procambium communication. Ethylene signaling is upregulated in *tdr* mutant
306 (Etchells *et al.*, 2012), which induces expression of transcription factors ETHYLENE
307 RESPONSE FACTOR1 (ERF1), ERF109 and ERF018. These ERF factors promote
308 procambial proliferation in a TDR-independent manner (Etchells *et al.*, 2012), forming a
309 compensatory loop for procambial maintenance. Given that ERf pathway also acts as a
310 compensatory mechanism for the TDR pathway (Etchells *et al.*, 2013; Uchida and Tasaka,
311 2013), ethylene signaling may be closely related to the ER signaling. Genetic interaction
312 between ethylene and ER signaling pathways was also reported in leaf hyponastic growth
313 (van Zanten *et al.*, 2010), implying that similar signaling networks may commonly work
314 for distinct events.
315 Auxin has been recognized to promote cambial activity in stems since as early
316 as 1930s (Snow, 1935). The auxin-dependent stimulation of cambial activity depends on
317 WUSCHEL-RELATED HOMEOBOX4 (WOX4), a transcription factor downstream of
318 TDR pathway (Hirakawa *et al.*, 2010; Suer *et al.*, 2011). Auxin upregulates the WOX4
319 expression, and TDR is required for the auxin-dependent WOX4 induction, suggesting a
320 crosstalk between the auxin signaling and TDR pathway to regulate WOX4 expression.
321 On the other hand, it was reported that *wox4 er* mutant did not show obvious vascular
322 defects unlike *tdr er* that exhibits a severe defect (Uchida and Tasaka, 2013), suggesting
323 that a WOX4-independent mechanism(s) must mediate the cooperative action by ER and
324 TDR. Recently, it has been reported that glycogen synthase kinase 3 proteins (GSK3s) act
325 downstream of TDR but independently of *WOX4* (Kondo *et al.*, 2014). Therefore, ER
326 signaling may interact with the GSK3 pathway. Interestingly,
327 BRASSINOSTEROID-INSENSITIVE 2 (BIN2), a member of GSK3s which was first

identified as a component of brassinosteroid (BR) signaling, acts upstream of YDA and downstream of ERf in the regulation of stomatal development (Kim *et al.*, 2012). Because BIN2 is also reported to act in phloem cells (Anne *et al.*, 2015) where ER proteins function for vascular development, it is plausible that BIN2 pathway modifies ER signaling in phloem. Furthermore, studies on hypocotyl elongation and lateral root development showed that BIN2 could function as a hub between TDR, BR and auxin pathways (Vert *et al.*, 2008; Cho *et al.*, 2014). Since ERf modulates auxin responses in some tissues (Chen *et al.*, 2013; Chen and Shpak, 2014; Tameshige *et al.*, 2016), it will be important to further examine relationships between ERf, TDR, auxin and BR signaling pathways in vascular development.

GA acts as a mobile factor that promotes internode elongation and cambial proliferation in stems (Dayan *et al.*, 2012), and these developmental processes are also regulated by ERf as described above. GA also induces expansion of xylem area in the hypocotyl (Ragni *et al.*, 2011). It has recently reported that *ER* and *ERL1* redundantly suppress the premature expansion of xylem in the hypocotyl (Ikematsu et al., 2016). The increased xylem size in the *er erl1* hypocotyl appears to contradict the fact that the *er erl1* mutation attenuates procambial proliferation in stems. It is interesting to investigate how the ER-dependent regulatory modules are flipped over between these tissues. The involvement of ER in xylem development in the hypocotyl had also been previously proposed (Ragni *et al.*, 2011). The interpretation was based on the fact that the L.*er* accession, which carries a loss-of-function allele of the *ER* gene, shows enhanced enlargement of the hypocotyl xylem, while this phenomenon does not occur in the Lansberg-0 (La-0) accession in which ER is functional (Ragni *et al.*, 2011). However, a recent study characterizing a progeny of L.*er* x La-0 inter-accession crosses revealed that the *er* mutation is not linked to the xylem expansion phenotype of L.*er,* suggesting that a yet unknown locus other than the *ER* locus is responsible for the xylem phenotype (Ikematsu *et al.*, 2016). This finding is not surprising given that the parental line of the L.*er* is likely the La-1 (Zapata *et al.*, 2016) that is distinct from the La-0 based on polymorphism analysis (http://www.lehleseeds.com/cgi-bin/hazel.cgi?action=detail&item=12&template=note1.html). The identification of the responsible locus for the xylem phenotype in L.*er* may

provide novel insight into xylem development in the hypocotyl. ERf also plays a role in a GA-related event of vascular development in the hypocotyl. The hypocotyl xylem produces fiber cells in response to GA (Dayan *et al.*, 2012; Ikematsu *et al.*, 2016; Ragni *et al.*, 2011). Interestingly, ER and ERL1 redundantly interfere the GA-mediated fiber formation (Ikematsu *et al.*, 2016). While it is plausible to expect that some EPF/EPFL peptides act as ligands for ER and ERL1 in vascular development in the hypocotyl, such a ligand remains to be identified.

CK promotes cambial proliferation (Matsumoto-Kitano *et al.*, 2008; Nieminen *et al.*, 2008; Bartrina *et al.*, 2011; Tokunaga *et al.*, 2012). As mentioned in the previous section of this manuscript, it is possible that the ER signaling buffers CK responses in a context-dependent manner, while it is still obscure whether this idea is applicable to the vascular regulation. Also, a relationship between CK pathway and TDR pathway remains to be addressed.

Jasmonate and strigolactone are also reported as regulators for (pro)cambial regulation (Sehr *et al.*, 2010; Agusti *et al.*, 2011), though there is no report which connects the action of these hormones with ER or TDR signaling. One or some phytohormones mentioned above may mediate the cell-cell communication from phloem to procambium as downstream events of the activation of ERf.

5. Concluding thoughts

The same EPFL4/6-ER module regulates the two distinct developmental processes, stem elongation and procambial maintenance, raising a hypothesis that common downstream components may be involved in both processes. For example, the MAP kinase cascade and their regulators are candidates for such common components. At the same time, there should also be a mechanism(s) that separates outputs from the common signaling module depending on a developmental context.

There are other challenges and questions to be addressed. For instance, *epfl4 epfl6* exerts milder effects than *er* on both inflorescence growth and procambial maintenance (Uchida *et al.*, 2012; Uchida and Tasaka, 2013), raising a question whether there are additional agonistic and/or antagonistic EPF/EPFLs besides EPFL4/6. It was reported that expression profiles of several *EPF/EPFL* genes changes according to

environmental conditions (Richardson and Torii, 2013), implying that ER signaling could be modulated in response to environmental stimuli. Though it is still unknown whether the EPFL4/6 production also responds to environmental changes, it will be interesting to investigate relationships between ERf-related developmental processes, a variety of environmental cues and changes in expression profiles of *EPF/EPFL* genes including *EPFL4/6*. Furthermore, it was shown that the phloem-specific activity of ER rescued not only phenotypes in inflorescence growth and procambial maintenance but also other phenotypes of *er,* such as rounder leaves with short petioles and blades as well as extreme dwarfism of *er erl1 erl2* (Uchida *et al.*, 2012). While it is yet to be tested whether these processes could also be regulated by EPFL4/6, it is likely that EPFL4/6 and/or other EPF/EPFLs act on ERf at phloem cells to regulate such diverse developmental processes. These future studies will provide further insights into the molecular nature of EPF/EPFL-ERf signaling that bounces in and out of the phloem.

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Figure Legends

Figure 1

Roles for ER family receptors and EPF peptides in stomatal patterning.

(A) Schematic illustration of stomatal development. A part of protodermal cell population acquires the character of meristemoid mother cell (MMC). MMC executes an asymmetric cell division, giving rise to two daughter cells with different characters: a meristemoid and a stomatal-lineage ground cell (SLGC). A meristeoid cell undergoes stereotypical asymmetric divisions repeatedly for self-renewal and amplifying SLGC. When a meristemoid is committed to differentiation, its character changes to guard mother cell (GMC), and the cell deform its shape from triangle to round one. Lastly, GMC undergoes a symmetric division to form a pair of guard cells (GC) forming a stoma. SLGCs differentiate into pavement cells. The transcription factors SPEECHLESS (SPCH), MUTE and FAMA form heterodimers with SCREAM (SCRM) or SCRM2, playing essential roles in the respective key steps as indicated by thin arrows.

(B) The phenotype of *er erl1 erl2* mutant on epidermal differentiation. The mutant shows increased and clustered formation of stomata.

(C) EPF2 peptides secreted from early stomatal lineage cells (MMCs and meristemoids) are perceived by a receptor complex consisting of ER, TMM and SERKs in neighboring protoderm cells, which prevents the protoderm from entering into stomatal lineage. ERL1 and ERL2 also participate in this step as minor but significant factors playing a redundant role with ER. EPFL9/STOMAGEN peptides secreted from mesophyll cells bind to ERf competitively against EPF2, blocking the EPF2 signaling. The MAP kinase cascade activated by the EPF2-receptor complex destabilizes SPCH protein, preventing the cell from acquiring cell fate of MMC.

(D) EPF1 peptides secreted from later stomatal lineage cells (late meristemoids and GMCs) are perceived by a receptor complex consisting of ERL1, TMM and SERKs in neighboring merostemoids, which prevents the merostemoid from producing stomata next to the existing one. ER and ERL2 also participate in this step as minor but significant factors playing a redundant role with ERL1. EPFL9/STOMAGEN may interfere the EPF1 signaling.

Figure 2

Roles for ER family receptors and EPFL peptides in inflorescence development.

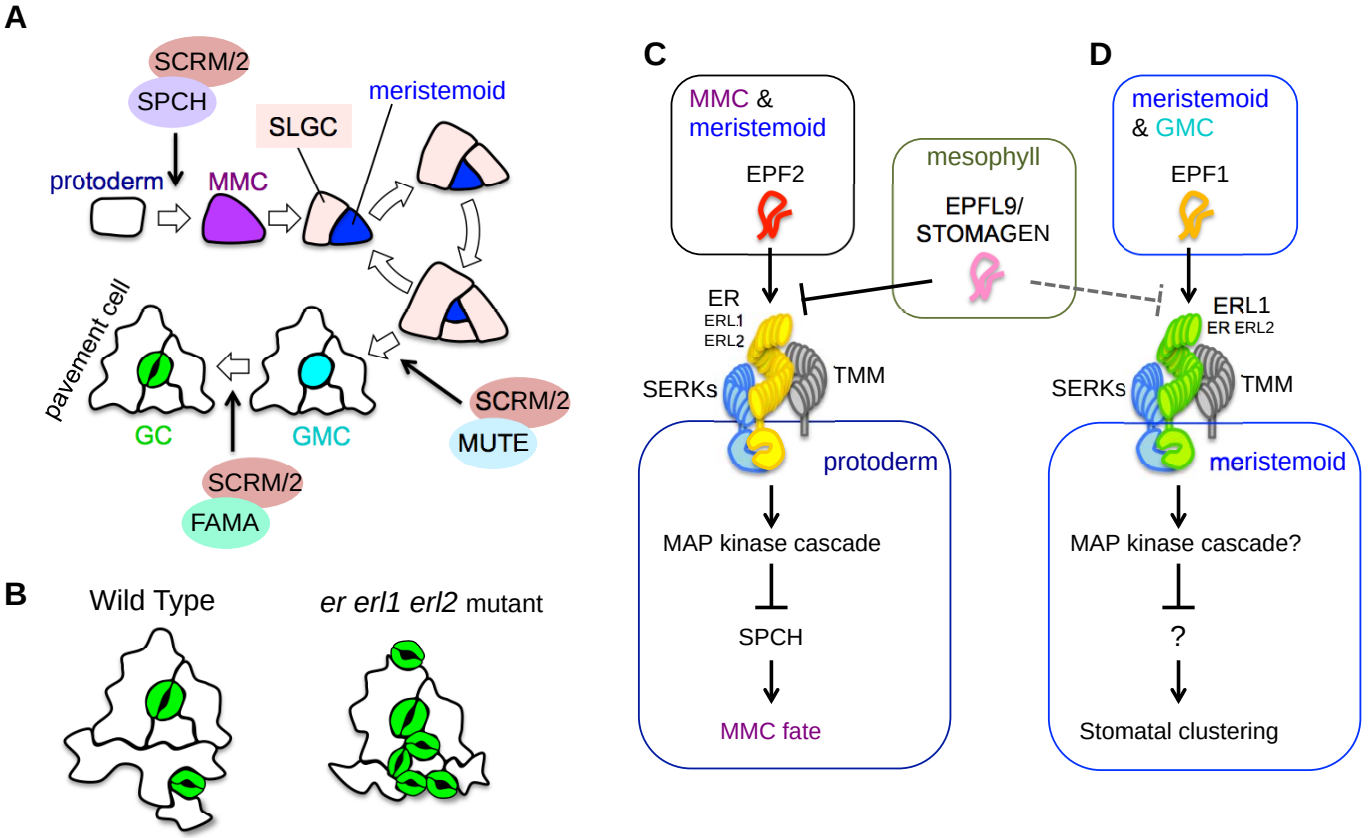
(A) The phenotype of *er* mutant on stem elongation. Inflorescence architectures and vertical stem sections are illustrated. The mutant shows short internodes and pedicels with decreased number of cortex cells. Cortex cells in the mutant also show abnormal enlargement.

(B) The phenotype of *er erl1* mutant on procambial maintenance. The mutant has less procambial cells than the wild type. Occasionally, some phloem cells directly contacts xylem cells without intervening procambial cells.

(C) Current model for action of ER receptor and EPFL peptides in stem elongation. EPFL4 and EPFL6 peptides secreted from endodermis of stems are perceived by ER in phloem, which activates stem elongation accompanied by cortex cell proliferation through unknown secondary cell-cell communication. In the schematic illustration of a vascular bundle, epidermis, cortex, endodermis, phloem, procambium (or cambium), xylem and pith tissues are arranged from the outside to the inside. Sieve elements and companion cells are formed in phloem. Vessel is produced in xylem.

(D) Current model for action of ER family receptors and EPFL peptides in procambial maintenance. Endodermis-derived EPFL4 and EPFL6 are perceived either by only ER or by both of ER and ERL1 in phloem. Another unknown ligand may also activate both ER and ERL1 or it may activate only ERL1. The molecular nature of phloem-derived secondary signals downstream of ERf signaling is unknown. TDIF peptides secreted from phloem are perceived by TDR/PXY in procambium. ER and TDR signaling pathways act in parallel for procambial maintenance.

Fig. 1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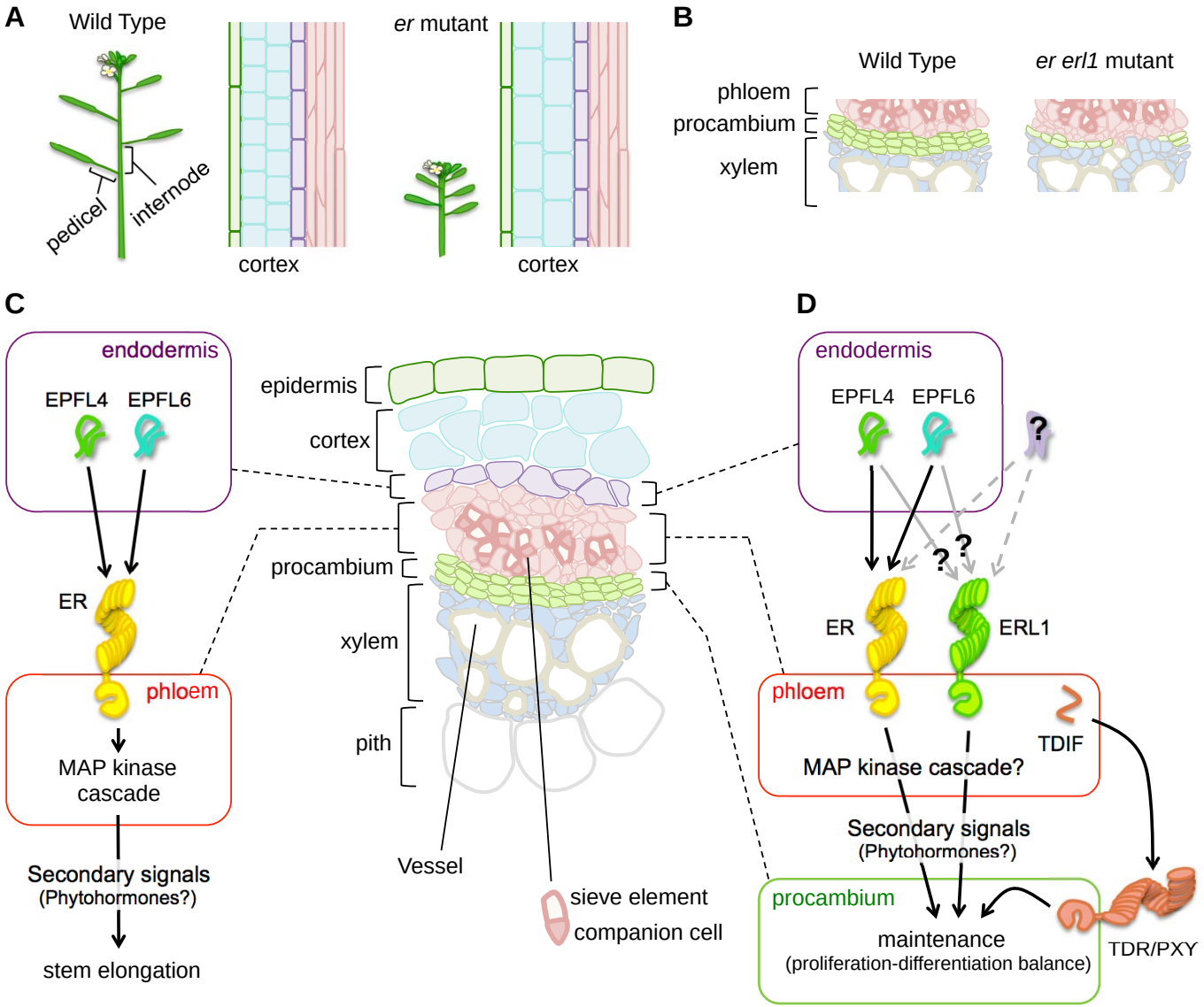
Stomatal clustering

Fig. 2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procambium
xylem
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endodermis
EPFL4
EPFL6
ER
phloem
MAP kinase cascade
Secondary signals (Phytohormones?)
stem elongation
epidermis
cortex
procambium
xylem
pith
Vessel
sieve element
companion cell
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endodermis
EPFL4
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ER
ERL1
phloem
TDIF
MAP kinase cascade?
Secondary signals (Phytohormones?)
procambium
maintenance (proliferation-differentiation balance)
TDR/PXY