

The secretory peptide gene *EPF1* enforces the stomatal one-cell-spacing rule

Kenta Hara,^{1,4} Ryoko Kajita,^{1,4} Keiko U. Torii,² Dominique C. Bergmann,³ and Tatsuo Kakimoto¹

¹Department of Biological Science, Graduate School of Sciences, Osaka University, Toyonaka, Osaka 560-0043, Japan; ²Department of Biology, University of Washington, Seattle, Washington 98195, USA; ³Biological Sciences, Stanford University, Stanford, California 94305, USA

Stomata are innovations of land plants that allow regulated gas exchange. Stomatal precursor cells are produced by asymmetric cell division, and once formed, signal their neighbors to inhibit the formation of stomatal precursors in direct contact. We report a gene of *Arabidopsis thaliana*, EPIDERMAL PATTERNING FACTOR 1 (*EPF1*) that encodes a small secretory peptide expressed in stomatal cells and precursors and that controls stomatal patterning through regulation of asymmetric cell division. *EPF1* activity is dependent on the TOO MANY MOUTHS receptor-like protein and ERECTA family receptor kinases, suggesting that *EPF1* may provide a positional cue interpreted by these receptors.

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Although multicellularity evolved independently in animals and plants (Baldauf 2003), both utilize asymmetric cell division for creating new cell lineages during development (Scheres and Benfey 1999; Betschinger and Knoblich 2004). In plants, stomatal development offers an excellent, tractable system to study asymmetric cell division. Stomata consist of two guard cells and a pore between them for gas exchange. The guard cells are produced through a series of asymmetric and symmetric cell divisions that not only produce the differentiated cells, but control the overall density and pattern of stomata on the organ surface (Bergmann 2003). In most dicot leaves, stomata follow a "one-cell-spacing" rule in which two stomata are separated by at least one intervening non-stomatal epidermal cell (Sachs 1991). This spacing is hypothesized to be important for efficient gas exchange (Nadeau and Sack 2002b). The first morphologically discernible stomatal precursor cell is the meristemoid, which is the smaller, triangular-shaped daughter cell produced by asymmetric cell division. The larger daughter cell may differentiate into a nonstomatal epidermal cell (pavement cell) or it may undergo its own asym-

metric cell division, creating a satellite meristemoid. A meristemoid has a self-renewing capability and can continue asymmetric divisions, but eventually converts into a guard mother cell (GMC), which then divides symmetrically to form two guard cells that constitute a stoma. The major enforcer of the one-cell-spacing pattern appears to be a signal sent from stomata and precursors (guard cells, GMCs, and mature meristemoids) to undifferentiated neighbor cells that influences the plane of cell division (Geisler et al. 2000).

A number of key regulators of the asymmetric cell divisions that ensure the one-cell-spacing rule and control stomatal densities are known. Mutations in *TOO MANY MOUTHS* (*TMM*) (Yang and Sack 1995; Geisler et al. 2000; Nadeau and Sack 2002a), *STOMATAL DENSITY AND DISTRIBUTION1* (*SDD1*) (Berger and Altmann 2000; von Groll et al. 2002), *YODA* (*YDA*) (Bergmann et al. 2004), and a triple mutant of *ERECTA* (*ER*); *ERECTA-LIKE1* (*ERL1*); *ERL2* (Shpak et al. 2005) result in plants with excess and clustered stomata. *TMM* is a membrane-anchored protein with an extracellular leucine-rich repeat (LRR) domain (LRR receptor-like protein; LRR-RLP), but no cytoplasmic signaling domain (Nadeau and Sack 2002a). *ER*, *ERL1*, and *ERL2* are LRR receptor kinases (LRR kinases) (Shpak et al. 2005). *TMM* and *ER* family proteins have been proposed to function together in the stomatal lineage to perceive positional information in relation to pre-existing stomata and precursors (Ingram 2005; Shpak et al. 2005). Because *TMM* mutations appear to affect only stomatal development, whereas the *ER* family is important for a broader range of biological processes (Shpak et al. 2004), it may be that a presumed *TMM/ER* family complex would rely on *TMM* for specificity of the positional information and the kinase-containing *ER* family proteins to transmit signals to downstream elements. One potential downstream target is *YDA*, a mitogen-activated protein kinase kinase kinase (MAPKKK) that has been shown to act as a switch between stomatal and pavement cell fate (Bergmann et al. 2004). The extracellular-localized subtilisin protease *SDD1* was proposed to process a precursor of an unknown mobile signal that controls stomatal density, and to a lesser extent, stomatal pattern (Berger and Altmann 2000). These cell-cell signal(s) are thought to ultimately affect the transcription factors that direct cell stomatal cell division and cell-type differentiation (Lai et al. 2005; Ohashi-Ito and Bergmann 2006; Macalister et al. 2007; Pillitteri et al. 2007).

The cellular mechanism of stomatal pattern and the molecular identities of the previously identified mediators strongly suggest that local activation of a signaling pathway in cells neighboring stomata or precursors regulates placement of meristemoid-forming division planes. Missing from the previous models was the identity of the (presumed proteinaceous) activating signal. In this manuscript we present evidence that the *EPIDERMAL PATTERNING FACTOR 1* (*EPF1*) gene encodes a protein capable of being such a signal.

Results and Discussion

Identification of the *EPF1* gene

In a genome-scale project to identify novel secreted peptide mediators that regulate plant development, we

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⁴These authors contributed equally to this work.

Corresponding author.

E-MAIL kakimoto@bio.sci.osaka-u.ac.jp; FAX 81-6-68505421.

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assayed the effect on *Arabidopsis* development of individually overexpressing 153 genes predicted to encode small (<150-amino-acid) secreted peptides. Through this screen, we identified a gene (At2g20875) that decreased stomatal density when overexpressed using a modified Cauliflower Mosaic Virus 35S promoter (Promoter_{2x35S- Ω}) (Fig. 1A,B). We designated the gene *EPF1*. The extent to which stomatal density decreased was correlated with

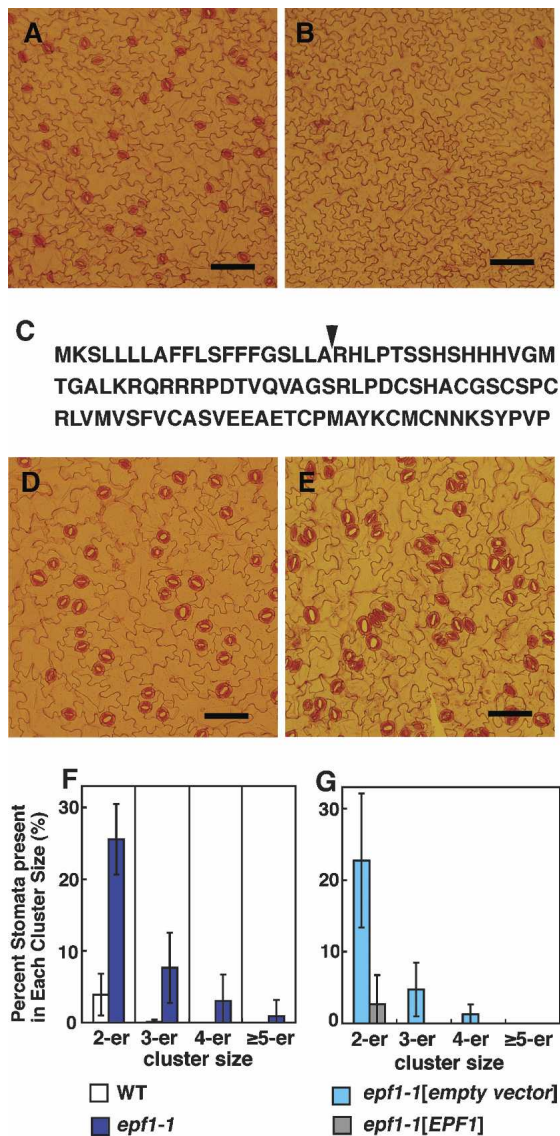


Figure 1. The *EPF1* gene, encoding a secretory protein, regulates stomatal patterning. (A,B) Overexpression of *EPF1* results in decreased number of stomata. An abaxial side of a cotyledon of *Arabidopsis* transformed with the pTK016 vector (A) and Promoter_{2x35S- Ω} -*EPF1* (B) are shown. After 7 d selection on BASTA (phosphinotricin) plate, resistant plants were further grown on nutrient plates for 7 d. (C) Deduced amino acid sequence of *EPF1* product. The predicted cleavage site flanking the N-terminal signal sequence is shown with an arrowhead. (D,E) *epf1-1* has clustered stomata. Abaxial sides of 20-d-old wild-type (D) and *epf1-1* (E) cotyledons are shown. (F,G) Percent stomata (mean \pm SD) present in stomatal clusters. (F) White column shows wild type and blue column shows *epf1-1*. (G) Light-blue column shows *epf1-1* transformed with the control vector, and gray column shows *epf1-1* transformed with the *EPF1* gene. Bars, 100 μ m.

the expression levels of *EPF1*, with the most severely affected plants producing no stomata (Supplementary Fig. S1). Such plants were significantly smaller than wild type, and were infertile. Transgenic lines with lower *EPF1* overexpression levels and only 10%–20% of the wild-type numbers of stomata did not exhibit any other discernable developmental phenotypes, suggesting that *EPF1* overexpression specifically affects stomatal development, and that the poor growth and infertility are secondary physiological consequences of a lack of stomata.

The deduced coding region of the *EPF1* product is 104 amino acids long, and is predicted to have an N-terminal secretory signal peptide, followed by a cleavage site between the 20th and 21st amino acid residues (Fig. 1B; SignalP 3.0 program, <http://www.cbs.dtu.dk/services/SignalP>; Nielsen et al. 1997). Outside of the signal peptide, *EPF1* contains no significant similarity to proteins (or protein domains) of known function.

Loss-of-function *epf1* mutant has a defect in one-cell-spacing rule of stomatal pattern

The overexpression phenotypes of *EPF1* suggest that this protein is capable of influencing stomatal pattern. We next sought to determine whether *EPF1* was required for normal stomatal development. For this purpose, a T-DNA insertion allele of *EPF1* (*epf1-1*) was characterized. In the homozygous mutant, both an increase in stomatal density and violations of the one-cell-spacing rule (clustering of stomata) were observed (Fig. 1D–F; Supplementary Table 1; Supplementary Fig. S3). The *epf1-1* mutant was complemented with a 3.3-kb genomic region spanning At2g20875 (Supplemental Material), indicating that the phenotype is due to disruption of this locus (Fig. 1G). *epf1-1* had no appreciable phenotypes unrelated to stomatal development, again indicating the specificity of *EPF1* in stomatal patterning. Stomatal clusters were most evident in cotyledon and rosette leaves, but clustering occurred in all stomata-producing organs examined (Supplementary Table 1). The clustered phenotype suggests that, in the absence of wild-type *EPF1*, the orientation of cell divisions in the stomatal lineage is incorrect. Indeed, we observed formation of new meristemoids adjacent to guard cells or precursors (Supplementary Fig. S2).

EPF1 is expressed in the stomatal precursor cells

Stomatal development occurs only in the aerial portions of the plant and predominantly in young leaves. *EPF1* transcript was detected in the shoot, but not the root, by RT-PCR (Fig. 2A). RNA gel blot analysis using RNA isolated from different developmental stages revealed that the *EPF1* transcript was highly expressed in developing leaves, contemporaneous with *TMM* and *SDD1* expression (Fig. 2B). We examined the cell-type specificity of *EPF1* expression by in situ RNA hybridization. In the epidermis of immature leaves, *EPF1* signal was detected in a punctuate pattern (Fig. 2C). In the 2- to 3-mm leaves, stomatal development is completed in the distal region. In such leaves, the punctuate *EPF1* signal was detected only in the proximal region, where stomatal precursor cells exist (data not shown). The signal pattern was similar to that of *SDD1*, which was reported to be expressed in stomatal precursor cells (von Groll et al.

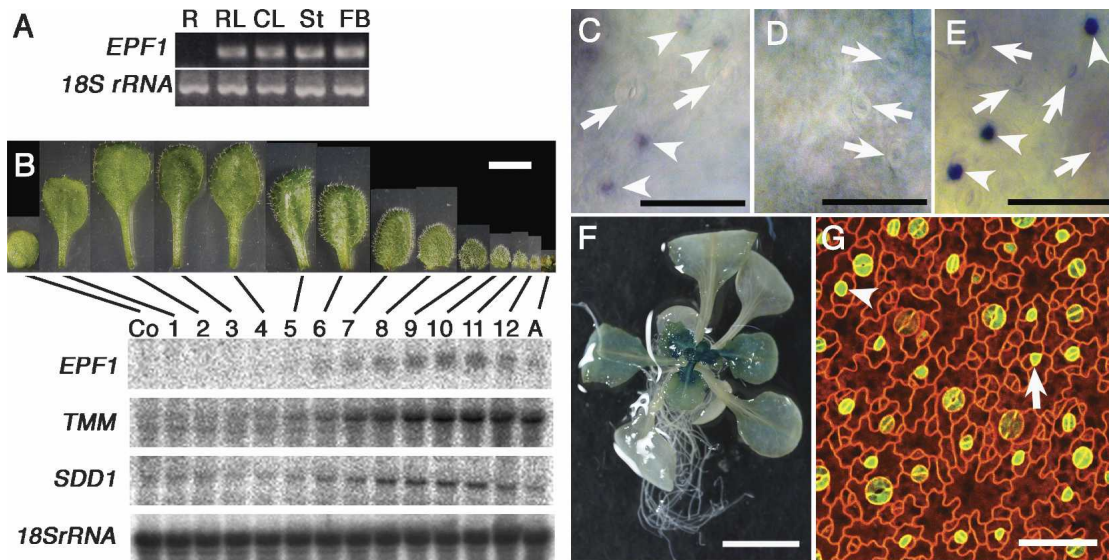


Figure 2. Expression of *EPF1*. (A) RT-PCR analysis for *EPF1* using RNA from roots (R), rosette leaves (RL), cauline leaves (CL), stems (St), and floral buds (FB). *18S rRNA* was used as a control target. (B) RNA gel blot analysis using RNA from cotyledons (Co), rosette leaves of different developmental stages (1–12), and shoot apex of 16-d-old wild-type plants. *EPF1*, *TMM*, *SDD1*, and *18S rRNA* were used as probes. (C–E) In situ hybridization for abaxial side of rosette leaves, probed with antisense *EPF1* (C), sense *EPF1* (D), and antisense *SDD1* (E). Arrows indicate mature stomata and arrowheads indicate hybridization signal. (F) *GUS* expression in 14-d-old *Arabidopsis* transformed with Promoter_{*EPF1*}-*GUS*. (G) Expression of Promoter_{*EPF1*}::GFP in abaxial rosette leaf. To highlight the outline of cells, leaves were stained with FM4-64, which accumulates in membranes (red color). The arrow and arrowhead indicate a meristemoid and a GMC, respectively. Bars: B, F, 5 mm; C–E, G, 50 μm.

2002), although *EPF1* signal was weaker than that of *SDD1* (Fig. 2C,E). Because outlines of cells in the whole-mount in situ hybridization samples were unclear, to

better understand the expression pattern we created a β-glucuronidase (*GUS*) fusion to the *EPF1* promoter (Promoter_{*EPF1*}-*GUS*) and green fluorescent protein (GFP)

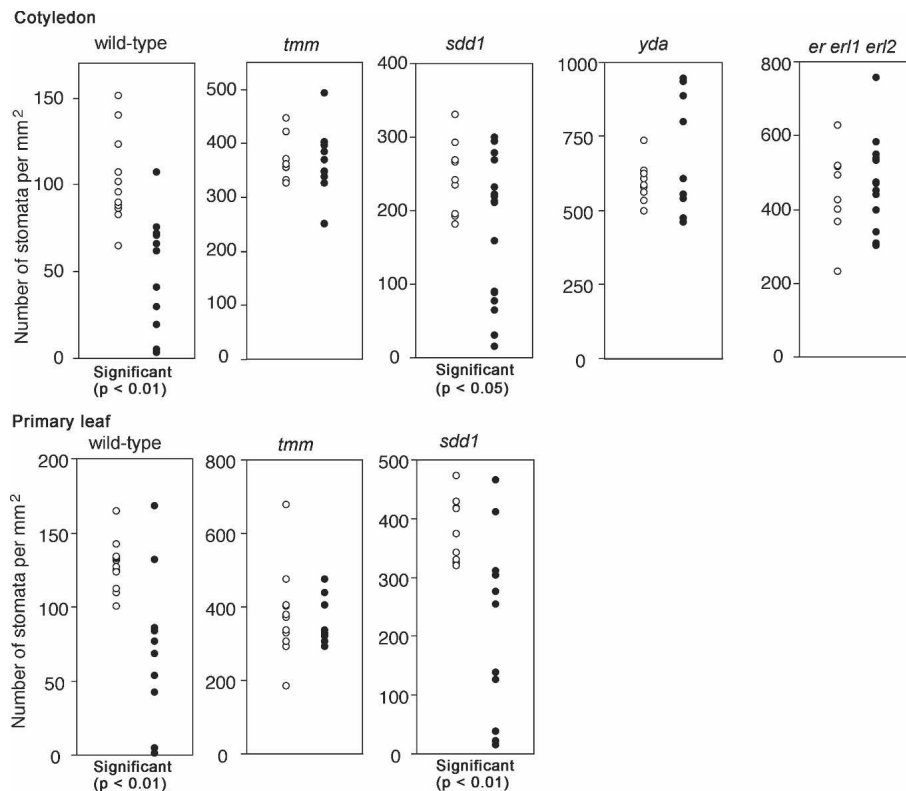


Figure 3. Effect of *EPF1* overexpression on stomatal density in different genetic backgrounds. Spots represent stomatal densities of independent T1 plants that had been transformed with the control vector (open spots) and Promoter_{2x35S-Ω-EPF1} (closed spots). (Top) Abaxial side of cotyledons of 15-d-old plants. (Bottom) Abaxial side of primary leaves of 20-d-old plants.

fusion to the *EPF1* promoter (Promoter_{*EPF1*}-*eGFP*) and analyzed reporter expression in developing plants. The reporter signals are absent from the shoot meristem (data not shown), are first detectable in young leaves when the stomatal lineage initiates (Supplementary Fig. S4), and are restricted to meristemoids, GMCs, and young guard cells (Fig. 2F,G). *EPF1* signal in the in situ RNA hybridization experiment was undetectable in young guard cells; however, the GUS and GFP reporter signals were positive. This discrepancy in expression may be due to stability of the reporters or, alternatively, to limited accessibility of RNA probes in the whole-mount in situ RNA hybridization. Interestingly, in contrast to genes for putative receptors (TMM and ER family) that are also expressed in the stomatal lineage neighbor cells (that respond to positional cues) (8,13), all measures of *EPF1* expression indicate that EPF1 is only found in cells implicated as signaling sources.

EPF1 is genetically upstream of genes for TMM receptor-like protein, ER family receptor-like kinases, and the YDA MAPKKK

A simple unifying model for stomatal pattern generation would be that SDD1 cleaves and activates the EPF1 peptide, EPF1 serves as a ligand for TMM and the ER family (Ingram 2005; Shpak et al. 2005), and this positional cue is relayed through a YDA-dependent signaling cascade. This model was tested in a series of genetic experiments. Promoter_{2x35S-Ω}-*EPF1* decreases stomatal density (Fig. 3); this decrease in stomatal density is dependent on functional *TMM*, *ER* family genes and *YDA* because both the increased stomatal density and stomatal clustering phenotypes characteristic of *tmm-1*, *yda*, and *er;erl1;erl2* are epistatic to Promoter_{2x35S-Ω}-*EPF1* (Fig. 3). Somewhat surprisingly, the *EPF1* overexpression phenotype did not depend on *SDD1* (Fig. 3). These results suggest that *EPF1* functions upstream of *YDA*, *TMM*, and *ER* family genes, but acts independently from *SDD1*.

To further verify the epistatic relationships, we made *epf1;tmm* and *epf1;sdd1* double mutants. The degree of stomatal clustering and increase in stomatal density in *epf1;tmm* was similar to that in the *tmm* single mutant (Fig. 4A; Supplementary Fig. S3); in agreement with the idea that TMM is a receptor for EPF1. *TMM* is a negative regulator of stomatal formation in cotyledons and leaves, but is a positive regulator of stomatal formation in the stem (Yang and Sack 1995). Therefore, we also examined genetic interactions between *epf1* and *tmm* in the stem. The *epf1* mutation alone moderately increased the number of stomata in the stem (Supplementary Table 2; Supplementary Fig. S5), a phenotype opposite of *tmm* (Supplementary Fig. S5). The double *tmm;epf1* mutant lacked stomata in the stem, again indicating that *tmm* is epistatic to *epf1* (Supplementary Fig. S5). In contrast, the effects of *epf1* and *sdd1* mutations on clustering and density of stomata were additive (Fig. 4B; Supplementary Fig. S3), strengthening the conclusion that *EPF1* and *SDD1* function independently (Fig. 3). While these results suggest that SDD1 does not process the EPF1 ligand for use in stomatal patterning (von Groll et al. 2002), it is possible that SDD1 processes an yet unknown signaling molecule, or that SDD1 processes a different class of protein, such as TMM.

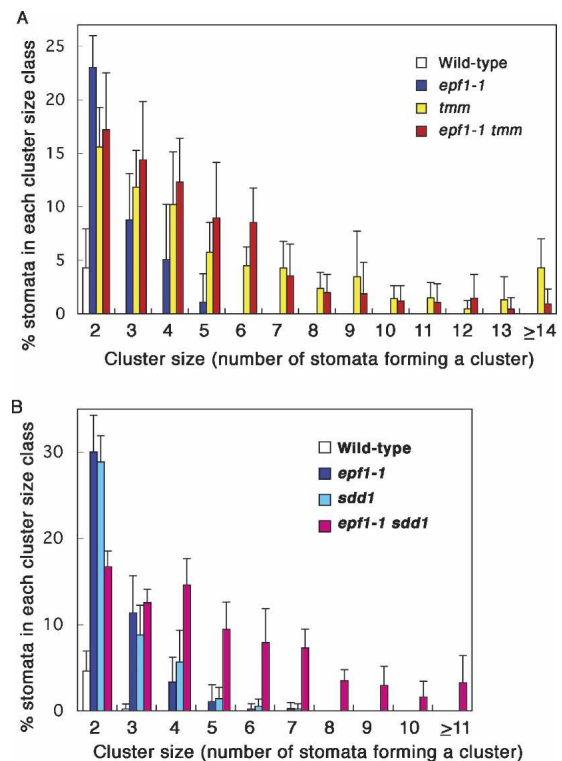


Figure 4. Size distribution of stomatal clusters. (A) *epf1*, *tmm*, and *epf1;tmm*. (B) *epf1*, *sdd1*, and *epf1;sdd1*. Single stomata are not shown.

Mutations in known stomatal patterning genes affect both stomatal density and clustering. However, the effects of mutations on stomatal density and the one-cell-spacing rule are different between affected genes: *TMM* is crucial for limiting density of stomata and for orienting spacing divisions. *SDD1* appears to have a primary role in controlling density and a secondary role in spacing (Nadeau and Sack 2003). *epf1-1* has a consistent defect in enforcing the one-cell-spacing pattern, yet its effect on stomatal density is moderate compared with *sdd1* or *tmm* (Supplementary Fig. S3; Supplementary Tables 1, 2), suggesting that *EPF1* has a primary role in orienting spacing divisions.

A model of stomatal patterning

We have shown that *EPF1* is expressed in the signaling cells of the stomatal lineage and influences stomatal density and pattern. The *EPF1* overexpression phenotype is dependent on functional *TMM* and *ER* family genes, and *tmm* is epistatic to *epf1*. This, together with the predicted secretory nature of the EPF1 product and receptor-like structure of TMM and ER family proteins is consistent with the hypothesis that EPF1 diffuses from stomatal precursors, is perceived by TMM and ER family receptors in neighbor cells and provides the positional information to regulate asymmetric cell division, which in turn, results in proper cell fate determination. Our results do not exclude the possibility that EPF1 has additional functions through TMM and ER family proteins to enforce pavement cell fate after the asymmetric cell division occurs, or to decrease cell division frequency of cells that contact stomata or precursors. The use of po-

sitional information to bias the orientation of asymmetric divisions is a universal theme in development (Horvitz and Herskowitz 1992; Scheres and Benfey 1999; Betschinger and Knoblich 2004). In animals ranging from *Caenorhabditis elegans* to *Drosophila* and vertebrates, positional information—well-known positional cues for asymmetries during cell division—are the Wnt-Frizzled family of signals and receptors—is translated through a set of conserved proteins into establishment of cell polarity (Goldstein et al. 2006). Plants achieved multicellularity independent of the animal lineages, and while both accomplish cell division and cell-type differentiation, the plant cells face constraints by the cell walls during development. Recruitment of a unique set of signaling molecules and receptors may have reflected the innovation by plants to orient asymmetric cell divisions and coordinate pattern formation.

Materials and methods

Plant materials and growth conditions

Arabidopsis ecotype Columbia was used in all experiments unless noted. Plants were grown in plates with GM medium (MS salts, 1% sucrose, 1/100 volume of 2.5% MES-KOH at pH 5.7, 0.3% Phytigel) under continuous light at 22°C.

Mutants used in this study are as follows: *yda-Y295* (Bergmann et al. 2004), *erl1-2*, *erl2-1* (Shpak et al. 2004), *tmm* (SALK_011959), *sdd1* (GABI_627-D04), and *epf1-1* (SALK_137549). In *epf1-1* (SALK_137549), the T-DNA is inserted 10 base pairs (bp) upstream of the translational initiation codon. The sequence around the T-DNA insert is as follows: 5'-cgtccgcaatgtgtattatcgtgtctctattcttctgtttcATAATA TATCATGAAGTCTCTTCTTCTCCTTGCCCTTTTCC-3'. In this sequence, the T-DNA region is shown with lowercase letters, the *Arabidopsis* genome sequence is shown with capital letters, and the translation initiation codon of *EPF1* is underlined. In *tmm* (SALK_011959), a T-DNA is present immediately upstream of the 518th nucleotide of the coding region of the single-exon gene *TMM*, with its LB flanking the 518th nucleotide. In *sdd1* (GABI_627-D04), a T-DNA is present immediately upstream of the 137th nucleotide of the coding region of the single-exon gene *SDD1*, with its LB flanking the 137th nucleotide. Double homozygous mutants were selected from offspring of plants generated by crossing.

Screen for bioactive secretory peptides

A collection of all genes of *Arabidopsis* coding for small proteins (<150 amino acids in length) was analyzed using the PSORT program to predict the destination of the gene products. A set of genes coding for products predicted to enter the secretory pathway was selected for further study. Entire coding regions of a set of genes were PCR-amplified and cloned in the *Sma*I site of a plant expression vector, pTK014. pTK014 was made by replacing the *GUS* gene and the *NOS* terminator of binary vector pGPTV-Kan (Becker et al. 1992) with duplicated *CaMV35S RNA* gene enhancers, the *CaMV 35S RNA* gene minimum promoter, and the Ω leader sequence from pE2113 (Mitsuhashi et al. 1996), a multicloning site including the *Sma*I site and *CaMV 35S RNA* gene terminator. One-hundred-fifty-three genes were transformed into *Arabidopsis* Columbia ecotype for overexpression. For most clones, >10 independent T1 transformants were examined for phenotypes. Through this screening, we found At2g20875 (*EPF1*), which caused a decrease in stomatal density when overexpressed. Subsequently, At2g20875 was also identified in the stomatal-enriched population in microarray data sets (Bergmann et al. 2004), and as a gene coexpressed with regulatory genes of stomatal development using Microarray data set (AtGenExpress Tissue Set analyzed by Expression Angler; http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi) (K.U. Torii, unpubl., data not shown).

In situ RNA hybridization

Whole-mount in situ RNA hybridization was performed using digoxigenin-labeled cRNA probes and alkaline phosphatase-labeled anti-digoxigenin, following the procedures outlined in Hejatko et al. (2006).

Plasmid constructs used in this study

For overexpression of *EPF1*, genomic sequence was PCR-amplified with the primers K148 (5'-AAAATGAAGTCTCTTCTCCTTGCCCTT-3') and K149 (5'-AAGGAAAACAAAACGGTTGAATGCATAGA-3'). The underlined AAA sequence preceding the translation initiation codon ATG in K148 was added to improve translational efficiency because it matches the most frequent sequence preceding translational initiation codons in *Arabidopsis*. The amplified fragment was cloned into pTK014 (giving kanamycin resistance to plants) or a similar vector, pTK016 (BASTA resistance in plants). The resulting constructs—pTK014-*EPF1* and pTK016-*EPF1*—were transformed into *Arabidopsis* using the flower-dipping method (Clough and Bent 1998).

For complementation of *epf1-1*, a 3308-bp genomic sequence consisting of a 2390-bp promoter, the entire coding region, and 517 bp of 3' region was PCR-amplified from the genome of wild-type Columbia using primers no. 2731 (5'-CCGCTCGAGTGGTTATGAAATATTTTCCCACTT-3') and no. 2786 (5'-CCCGAGCTCTAATTTTATTTTATTTTGTTGTAGAGGAGA-3'). Underlined regions introduce *Xho*I and *Sac*I sites. The amplified fragment was digested with *Xho*I and *Sac*I, and cloned between the *Xho*I and *Sac*I sites of pGWB1, which is a binary vector with Hygromycin-selectable marker in plants. The resulting plasmid was transformed into plants homozygous for *epf1-1*.

For construction of Promoter_{*EPF1*}-*erGFP*, a 2457-bp upstream region of *EPF1* was PCR-amplified with the primers K176 (5'-ACGACGATGTCCTCTTTTGTCTTTGAGAA-3') and K197 (5'-CCGCTCGAGGATATATTATCGCAAGTGGTAAAAGTAA-3'); the underlined region indicates an added *Xho*I site, and cloned between the *Sma*I and *Xho*I sites of pRK2, which carries *erGFP* (GFP with ER targeting signal). For construction of Promoter_{*EPF1*}-*erGFP*, a 2457-bp upstream region of *EPF1* was PCR-amplified with the use of the primers K176 (5'-ACGACGATGTCCTCTTTTGTCTTTGAGAA-3') and K177 (5'-TGATATATTATCGCAAGTGGTAAAAGTAA-3') and blunt-end-cloned in the *Sma*I site of pGPTV-Kan (Becker et al. 1992).

Phenotypic analysis

For analysis of the stomatal pattern, plants were fixed in 90% ethanol/10% acetic acid, washed in water, stained with 1 μ g/mL Safranin O, washed with water, then examined with a confocal microscope (FV300, Olympus) using an excitation wavelength of 543-nm and emission spectrum of 570-nm light.

For determining the number of stomata in each cluster size and stomatal density, four square areas of 0.49 mm² were counted per organ, and the numbers were averaged to yield an estimated cluster distribution per organ. Organs from at least six plants were used for each genotype.

For quantitative examination of the stem, pedicel, and silique, and for images in Figure 1, the epidermis of fixed samples was peeled and stained with 1 mg/mL Safranin O, and then examined under a transmission light microscope. Images of *EPF1* expression were obtained by examining plants expressing Promoter_{*EPF1*}-*erGFP* (488-nm Argon laser excitation, 510- to 530-nm emission) and counterstaining with a membrane marker FM4-64 (10 μ g/mL) (488-nm Argon laser excitation, >570-nm emission).

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