Supplementary Methods

Plant materials and growth conditions

The *Arabidopsis* accession Columbia (Col) was used as wild type. All plants used in this study are in Col background. The following mutants and transgenic plant lines were reported previously: *er-105, erl1-2, erl2-1* and their higher order mutants [1]; *tmm-1* [2]; *epf1, epf2, epf1 epf2* and *tmm-KO* [3, 4]; *ERΔK* in *er-105*, *ERL1ΔK* in *erl1-2,* [5]; and *STOMAGEN-ami* line (line 12; a gift from Prof. Ikuko Hara-Nishimura)[6]. The *STOMAGEN*-ami lines were introduced into various mutant or transgenic backgrounds via genetic crosses. Plants were grown as described previously [7]. For phenotypic analysis, seedlings were grown on ½ Murashige and Skoog (MS) media containing 1x Gamborg Vitamin (Sigma), 0.75 % Bacto Agar, and 1 % w/v sucrose under continuous light condition at 50 □mole s^{-1} m^{-2} (light intensity measured by LI-250A; LI-COR).

Plasmid construction and transgenic plants generation

pKUT608 (pENTR-*STOMAGEN*) and pKMP127 (*Est::STOMAGEN*) were generated. See Supplemental Table S1 for plasmid and primer sequence information. Transgenic Arabidopsis plants were generated by the floral dip method[8]. Multiple transgenic lines per construct were subjected to phenotypic characterization and representative lines (3 lines if lines were established, and 12-14 lines if T1 lines were used) were used for quantitative analyses. The *Est::STOMAGEN* lines were introduced into various mutants or transgenic backgrounds via genetic crosses.

Chemical induction of transgene

Transgenic Arabidopsis seedlings carrying *Est::STOMAGEN* was germinated on ½ MS medium supplemented with 10 μM estradiol (Sigma). Induction of *STOMAGEN* gene expression (*iSTOMAGEN*) was confirmed by RT–PCR (see Extended Data Fig. 2). The induction was further confirmed by observing the epidermal phenotypes of cotyledons and hypocotyls using a confocal microscope.

RT-PCR analysis

RNA extraction, cDNA synthesis, and RT-PCR were performed as previously described [9]. For a list of primers, see Extended Data Table 1.

Histology, microscopy and image analysis

Confocal microscopy images were taken using either Zeiss LSM700 operated by Zen2009 (Zeiss) described previously [5] or Leica SP5-WLL operated by LAS AF (Leica). Cell outlines were visualized with either propidium iodide (PI: Molecular Probes) or FM4-64 (Invitrogen) and observed using the HyD detector with excitation 515 nm, emission 623-642 nm. The images were false colored using Photoshop CS6 (Adobe). Clearing of seedlings by chloral hydrate and observation using differential interference contrast (DIC) microscope was performed as described previously [10]. For histological analysis, seedlings were stained with toluidine blue-O (TBO: Sigma) as the following. Briefly, samples were placed in 9:1 v/v ethanol to acetic acid overnight, rehydrated through reduced ethanol series to deionized water, then stained with 0.5 % TBO for 3 min. Seedlings were immediately rinsed with deionized water and subsequently mounted in 15 % v/v glycerol. For bright-field and differential interference contrast (DIC) microscopy, images were taken under Olympus BX51 equipped with DP73 digital camera operated by CellSens Standard software (Olympus).

Quantitative analysis of epidermis

Abaxial cotyledons from 10-day-old seedlings of respected genotypes were subjected to TBO staining or DIC microscopy. The central regions overlying the distal vascular loop were imaged and numbers of epidermal cells, stomata and their cluster size were quantified. For each genotype, sample size of 14-16 was used and over thousand epidermal cells were counted to provide statistical robustness. For cotyledons of *Est::STOMAGEN* lines, individual T1 seedlings were subjected to analysis. For hypocotyls, three representative T2 *Est::STOMAGEN* lines were analyzed. For each seedling, representative image was taken at the exact same location to minimize the variance. Specific numbers of stomata are listed for each genotype in corresponding figure legends. Statistical analysis (Wilcoxon Rank Sum Test for non-parametric text) was performed using R version 3.0.3 for SD, SI, and SLGCI. P values are indicated in each figure legend.

Transient protein expression in *Nicotiana benthamiana*

A. tumefaciens strain GV3101 was transformed with expression clones and grown in yeast extract and beef medium supplemented with relevant antibiotics. Bacterial cultures were precipitated and resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES (pH 5.6), and 150 μM acetosyringone). Culture densities were adjusted to an OD_{600} of 1.0, and the cells were incubated at room temperature for 4 h prior to infiltration. Equal volumes of cultures carrying different constructs were mixed. To enhance transient expression in tobacco, the silencing suppressor p19 (a gift from Professor Sir D. Baulcombe) was coinfiltrated [11]. The bacterial suspensions were infiltrated into young but fully expanded leaves of *N. benthamiana* plants. After infiltration, plants were cultivated at 25°C and collected for further biochemical assays after 48-72 h.

Peptide expression, purification, and refolding

Recombinant MEPF2 peptide was prepared as reported previously [5] and the mature Stomagen peptide, either wild-type or non-refolding mutant version in which all six cysteines were substituted by serines, were chemically synthesized (Invitrogen and BioSynthesis). The Stomagen peptide was dissolved in 20 mM Tris-HCl, pH8.8, and 50 mM NaCl and refolded (Mini dialysis kit, MWCO:1,000, GE Healthcare) for 3 d at 4°C using glutathione (reduced and oxidized forms; Wako) and L-arginine ethyl ester dihydrochloride (Sigma). The peptides were further dialyzed three times against 50 mM Tris-HCl, pH8.0 for 1.5 d to remove glutathione. For non-folding mutant Stomagen, chemically synthesized peptides were dissolved in 50 mM Tris-HCl, pH8.0. The resulting MEPF2, Stomagen, and mutant Stomagen peptides were fractionated using C18 column (Gemini) by HPLC (Waters Delta Prep 3000 HPLC) as previously described to determine the purity of each peptide[5]. The separated peaks were collected, and each peak was identified by MALDI-TOF mass spectrometry on an Autoflex II mass spectrometer in positive ion mode (Bruker Daltonics) using 2:1 a-cyano-4-hydroxycinnamic acid and 2,5-dihydroxy-benzoic acid matrix. The collected HPLC-purified MEPF2 and Stomagen peptide peaks were freeze-dried, then re-dissolved to appropriate concentration. Quantification of the active populations of peptides were determined using NanoDrop8000 (Thermo Scientific) using the following molar extinction coefficients: Stomagen, 5960; EPF2, 6460; LURE2, 23950 mol^{-1} cm^{-}

[1]. For bioassays, freeze-dried peptides were re-dissolved to appropriate concentration in MS medium. For subsequent biochemical assays, the amounts of bioactive peptides were calculated from this quantification.

Peptide bioassays

Refolded recombinant MEPF2 and Stomagen peptides in buffer were applied to 1 day-old Arabidopsis plants that had germinated on ½ MS medium. After 5 d of further incubation in ½ MS liquid medium containing each peptide (2.5-5 μM concentration), stomatal phenotypes of abaxial cotyledon epidermis were determined by inspection with a confocal microscope as described previously[5].

Immunoprecipitation, protein gel electrophoresis and immunoblots

For immunoprecipitation and co-immunoprecipitation assays, *N. benthamiana* leaves expressing CaMV35S::ERΔK-GFP, CaMV35S::FLS2ΔK-GFP, CaMV35S::TMM-GFP, CaMV35S::GFP, or empty vector were subjected to protein preparation (microsomal fraction enrichment for all except soluble GFP). Co-incubation with Stomagen (1 μM) or LURE2 peptides (1 μM) and immunoprecipitation procedure are described in the Ligand Competition Assays section below. Immunoprecipitation using either anti-GFP (Abcam ab290) antibodies and protein gel immunoblot (Western blot) analysis using anti-GFP (Invitrogen C163), anti-FLAG (Sigma-Aldrich M2), anti-His (Qiagen anti-His5 34660), and anti-Stomagen (a gift from Prof. Ikuko Hara-Nishimura)[6] antibodies were performed as described previously[5]. As secondary antibodies, either goat anti-mouse (GE Healthcare NA931) or anti-rabbit IgG horseradish peroxidase-linked antibodies (Sigma A6154) were used at a dilution of 1:50,000. The protein blots were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Quartz Crystal Microbalance

QCM measurements were performed using QCM-Z500 (KSV Instruments) and commercially available AT-cut polished QCM crystals with a fundamental resonant frequency of 4.95 MHz (International Crystal Manufacturing Co.) as reported previously [5]. The QCM crystal chips were pre-treated with anti-GFP antibody (Abcam ab290) to functionalize the chip surface. Subsequently, GFP-tagged receptors or GFP expressed in *N. benthamiana* and extracted as a

microsomal fraction (for ERΔK-GFP and TMM-GFP) or a total fraction (GFP) were immobilized onto a QCM sensor chip via anti-GFP antibody linkage. The chips were washed with a phosphate buffer extensively. After establishing a stable baseline using phosphate buffer solution, purified bioactive MEPF2 or Stomagen peptides in the phosphate buffer was added stepwise to the QCM chamber. The frequency change for QCM was monitored until no further change was observed, indicating equilibrium. All experiments were performed at 4°C in stop-flow mode. The peptide-receptor binding was quantified *via* QCM by measuring the frequency shifts, ΔF, at several peptide concentrations. To determine the dissociation constant (K_d) of each peptide-receptor pair, the experimental frequency shift values were fitted to the Langmuir adsorption model: $-\Delta F = \Delta F_{max}\, C / (C + K_d)$, where ΔFmax is the frequency shift when the binding is saturated and *C* is the concentration of the bulk solution, using a least squares regression.

Ligand competition assays

N. benthamiana leaves expressing *CaMV35S::ERΔK-GFP* were ground in liquid nitrogen and homogenized in extraction buffer (100 mM Tris-HCl pH 8.8, 150 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM NaF, 1 mM PMSF, 1:1,000 Complete protease inhibitor cocktail (Roche Applied Science)). The slurry was centrifuged at 10,000 *g* for 15 min at 4°C. The supernatant was sonicated on ice and then centrifuged at 100,000 *g* for 30 min at 4°C to give a pellet of the microsomal fractions. The pellet was resuspended in membrane solubilization buffer (100 mM Tris-HCl at pH 7.3, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM NaF, 1 mM PMSF, 1:1,000 Complete protease inhibitor cocktail) to release membrane proteins. The solution was sonicated on ice and centrifuged again at 100,000g for 30 min at 4°C. The supernatant was incubated with Protein G-coupled magnetic beads (Dynabeads Protein G, Invitrogen) that captured anti-GFP (ab290; Abcam) antibody at 4°C for 2h with gentle agitation. Then, the beads were washed four times with 500 μl of phosphate buffer (pH 7.4). The immunoprecipitates were suspended in 500 μl of binding buffer (50 mM MES-KOH, pH 5.5 with 100 mM sucrose) containing 1 μM MEPF2-MYC-HIS peptide in the absence or presence of different concentration of unlabeled bioactive Somagen peptide and then incubated at 4°C for 1h with gentle agitation. The reaction mixture was washed four times with 500 μl of phosphate buffer (pH 7.4) to separate bound and free MEPF2-MYC-HIS peptide, and precipitated proteins

were eluted with 2x SDS sample buffer at 80 °C for 5 minutes. Either total membrane or immunoprecipitated proteins were separated on a SDS-PAGE gel and transferred to PDVF membrane (Millipore) for immunoblot analysis using monoclonal anti-GFP (C163, 1:1,000, Invitrogen), anti-MYC (ab32, 1:1,000, abcam) or anti-Stomagen antibodies (1: 5,000, a gift from Prof. Ikuko Hara-Nishimura)[6] as primary antibodies. As secondary antibodies, either goat anti-mouse or rabbit IgG horseradish peroxidase-linked antibodies (GE Healthcare NA931; Sigma A6154) were used at a dilution of 1:50,000. Co-immunoprecipitated MEPF2 was detected first. Then, the same blot was re-probed with anti-Stomagen antibody to detect Stomagen.

Four biological replicates were performed and subjected to quantification of the IC50 values as the following. Band intensities on Western blots were quantified using IMAGEJ (http://rsb.info.nih.gov/ij/index.html). Pixel values were measured on equal-sized areas and normalized against the bands detecting same immmunoprecipitates by monoclonal anti-GFP antibody. The intensity values shown in the paper are the ratios relative to the references, and values were analyzed by non-linear regression analysis using OriginLab version 6 (OriginLab) to calculate the IC50 value.

MAPK phosphorylation assays

12-day-old Arabidopsis seedlings were grown for five days on ½ MS media plates and then transferred to ½ MS liquid media in a 12-well cluster plate (Falcon 3047®). Seedlings were treated with buffer only, MEPF2 (2.5 μM), or with Stomagen (5 μM) at room temperature before being pooled for harvest. For heat denaturation of MEPF2, the pepide solution was treated at 95 °C for two hours prior to MAPK phosphorylation assays and bioassays. Plant materials were ground in liquid nitrogen, and then extracted with buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 10mM Na_3VO_4, 10mM NaF, 50 mM β-glycerolphosphate, 1 mM PMSF, 1 tablet/50 ml extraction buffer of proteinase inhibitor mixture, 10% glycerol, 7.5% (w/v) PVPP). After centrifugation at 13,000 rpm for 30 min, the protein concentration was determined using a Bradford assay (Bio-Rad). Immunoblot analysis was performed using anti-phospho-ERK (1:2,000, Cell signaling) antibody as primary antibody, and peroxidase-conjugated goat anti-rabbit IgG (1:15,000, Sigma) as secondary antibody.

Supplementary References

1. Shpak, E.D., McAbee, J.M., Pillitteri, L.J. & Torii, K.U. Stomatal patterning and differentiation by synergistic interactions of receptor kinases. *Science* **309**, 290-293 (2005).
2. Nadeau, J.A. & Sack, F.D. Control of stomatal distribution on the Arabidopsis leaf surface. *Science* **296**, 1697-1700. (2002).
3. Hara, K., Kajita, R., Torii, K.U., Bergmann, D.C. & Kakimoto, T. The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. *Genes Dev* **21**, 1720-1725 (2007).
4. Hara, K. *et al.* Epidermal cell density is auto-regulated via a secretory peptide, EPIDERMAL PATTERNING FACTOR2 in Arabidopsis leaves. *Plant Cell Physiol* **50**, 1019-1031 (2009).
5. Lee, J.S. *et al.* Direct interaction of ligand-receptor pairs specifying stomatal patterning. *Genes Dev* **26**, 126-136 (2012).
6. Sugano, S.S. *et al.* Stomagen positively regulates stomatal density in Arabidopsis. *Nature* **463**, 241-244 (2010).
7. Shpak, E.D., Lakeman, M.B. & Torii, K.U. Dominant-negative receptor uncovers redundancy in the Arabidopsis ERECTA leucin-rich repeat receptor-like kinase signaling pathway that regulates organ shape. *Plant Cell* **15**, 1095-1110 (2003).
8. Clough, S.J. & Bent, A.F. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* **16**, 735-743 (1998).
9. Pillitteri, L.J., Peterson, K.M., Horst, R.J. & Torii, K.U. Molecular profiling of stomatal meristemoids reveals new component of asymmetric cell division and commonalities among stem cell populations in Arabidopsis. *Plant Cell* **23**, 3260-3275 (2011).
10. Pillitteri, L.J., Sloan, D.B., Bogenschutz, N.L. & Torii, K.U. Termination of asymmetric cell division and differentiation of stomata. *Nature* **445**, 501-505 (2007).
11. Voinnet, O., Rivas, S., Mestre, P. & Baulcombe, D. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* **33**, 949-956 (2003).